University of Alberta

CHARACTERIZATION OF ADHESIVE COMPLEXES IN A HUMAN SMALL CELL LUNG CARCINOMA (SCLC) CELL MODEL SYSTEM

By

ANITA J. GILCHRIST

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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submitted by

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in partial fufillment of the requirements for the degree of

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ABSTRACT

Small cell lung carcinoma (SCLC) is a significant clinical problem since it undergoes early and extensive metastatic dissemination. SCLC cells appear to arise by oncogenic transformation of self-renewing pulmonary neuroendocrine cells, which have the potential to differentiate into a variety of lung epithelial cell lineages. Epithelial-mesenchymal transitions (EMT) involved in such cell type conversions leads to the acquisition of an invasive and metastatic phenotype. Changes in the adhesive properties of cells during normal or pathological EMT are complex and involve multiple systems such as cadherin-mediated cell-cell adhesion and integrin-mediated cell-matrix adhesion. In order to investigate the contribution of adhesive mechanisms involved in such transitions, a SCLC cell line was exposed to 5-bromodeoxyuridine (BrdU). This treatment induced a dramatic conversion from non-substrate-adherent aggregates to monolayers of cells exhibiting an epithelioid phenotype, concurrent with a loss of tumorigenicity *in vivo*. Characterization of this model system *in vitro*, showed that the phenotypic transition was concomitant with downregulation of vimentin, upregulation of cytokeratins as well as redistribution of the actin cytoskeleton, and upregulation of cell-cell and cell-matrix adhesion molecules. Changes in the levels, organization and signaling functions of cell-cell and cell-matrix adhesive complexes may be critical for neoplastic progression and the eventual resistance of SCLC cells to therapy.

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LIST OF ABBREVIATIONS

α-cat	α-catenin
β-cat	β-catenin
γ-cat	γ-catenin
AJ	adherens junctions
ADF/cofilin	actin depolymerizing factor/cofilin
APC	adenomatous polyposis coli
APUD	amine precursor uptake and decarboxylation
ARF	ADP-ribosylation factor
ARP 2/3 complex	actin-related protein 2/3 complex
AVP	arginine vasopressin
BrdU	bromodeoxyuridine
cad	cadherin
CAMs	cell adhesion molecules
CHAPS	3-[3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CK	cytokeratin
CK-BB	creatine kinase bovine brain isoenzyme
co-IP	co-immunoprecipitation
CSK	cytoskeleton
DDC	L-dopa decarboxylase
DTR/ HB-EGF	diptheria toxin receptor/ heparin-binding EGF-like growth factor
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT/ MET	epithelia to mesenchyme/ mesenchyme to epithelia transitions
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERM	ezrin/radixin/moesin
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAP	GTPase activating proteins
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factors
GRP	gastrin releasing protein
GSK3-β	glycogen synthase kinase 3-β
GTP	guanosine triphosphate
HGFR	hepatocyte growth factor receptor
HRP	horseradish peroxidase
IGF-1	insulin-like growth factor
ILK-1	integrin-linked kinase
LEF/TCF-1	lymphoid enhancer factor/ T-cell factor
mAbs	monoclonal antibodies

MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C-kinase substrate
MDCK	Madine-Darby canine kidney cells
MMP	matrix metalloproteinases
MEK	MAP or extracellular signal-related kinase
MHC	major histocompatibility complex
NCAM	neural cell adhesion molecule
NE	neuroendocrine
NEB	neuroendocrine bodies
NSCLC	non-small cell lung carcinoma
NSE	neuron specific enolase
p120cat	p120 catenin
p130cas	Crk-associated substrate
PCD	programmed cell death
PH	pleckstrin homology
PI	phosphatidylinositol
PI3-K	phosphatidylinositol 3-kinase
PI4-K	phosphatidylinositol 4-kinase
PIP_2	phosphatidylinositol 4,5 biphosphate
PKC	protein kinase C
polyHEMA	polyhydroxyethylmethacrylate
PTB	phosphotyrosine-binding
PTK	protein tyrosine kinase
RAPD	randomly amplified polymorphic DNA
Rb	retinoblastoma
RGD	Arg-Gly-Asp
Rho	Ras-homologous
SCF	scatter factor
SCLC	small cell lung carcinoma
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
TCE	total cell extract
TGF- β1	transforming growth factor- β1
TJ	tight junctions
TRITC	tetramethylrhodamine-5(and6)-isothiocyanate
TSGs	tumor suppressor genes
VASP	vasodilator stimulated phosphoprotein

CHAPTER 1 INTRODUCTION

1.1 Lung cancer biology

Lung cancer is a significant medical problem both in North America and globally. Primary lung cancer is the second leading cause of deaths in the U.S. and accounts for approximately 14% of all new cancers (1). In women, lung cancer has surpassed breast cancer as the leading cause of cancer death since 1987 and is expected to account for 25% of all female cancer deaths in 2001. In men, lung and bronchus cancers are also estimated to be the leading cause of cancer deaths accounting for 31% of all cancer deaths in 2001 (2). Despite the prevalence of lung cancer only limited progress has been made in management and/or treatment of the disease (1). With a world-wide increase in cigarette smoking, which is known to cause 80-90% of lung cancers, it is likely that lung cancer will continue to be a difficult clinical problem (3). According to the World Health Organization classification, lung cancers can be classified into four major types: squamous cell carcinoma, adenocarcinoma, large cell (collectively referred to as non-small cell lung carcinoma or NSCLC) and small cell lung carcinoma (SCLC) (4,5). Historically, cancers of the lung have been classified on the basis of histological appearance using specific biomarkers to determine the type and degree of differentiation. In addition, cultured cell lines derived from the various types of lung cancer were found to closely resemble the cellular phenotype of the original tumors (6). Characterization and classification of these biological properties as indicators of malignant potential and response to therapy has practical importance in the diagnosis and prognosis of lung cancer.

1.1.1 Clinical, morphological and histological definition of SCLC

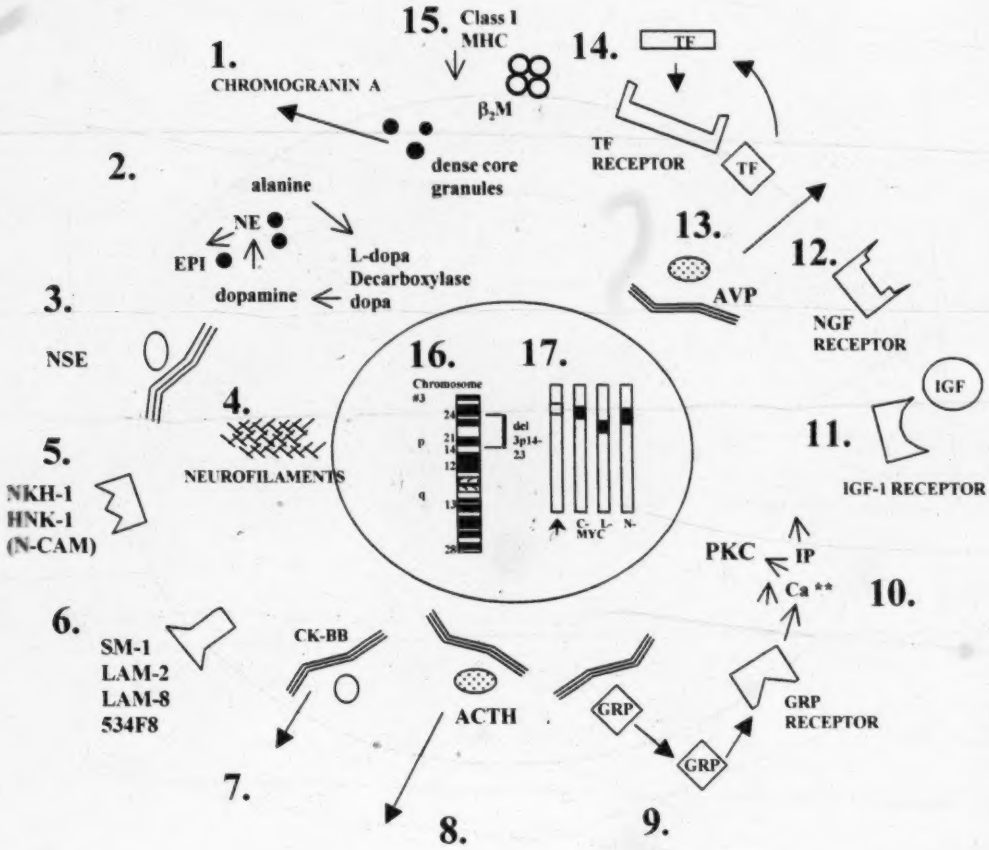
Within the lung cancer types, SCLC is a highly aggressive neoplasm, accounting for approximately 25% of cases (4,7,8). Although the majority of SCLC cells are initially sensitive to radio- and chemo- therapy, their inherent heterogeneity results in the eventual development of tumor cells which are resistant to standard chemotherapeutic agents. Thus, SCLCs undergo early, rapid, and extensive metastatic dissemination, are correlated with poor prognosis and have a low (< 5%) 5-year survival rate (1,2).

The morphology, ultrastructure, biochemistry and cytogenetics of SCLC have been well-characterized (Fig. 1) (9). The ability of SCLC cells to produce, secrete and respond to their own growth factors can create a positive feedback loop that leads to a decreased dependence on external factors, and stimulation of clonal growth in tumor cells (Fig. 1, 9-14). In fact, classic SCLC tumors characteristically exhibit a high growth fraction i.e. an increased number of cells able to undergo proliferation, as well as an increased rate of progression through the cell cycle. Efforts to identify and target biomarkers specific to SCLC tumor cells (Fig. 1, 6) have been hindered by the degree of antigenic and biological diversity exhibited. This extensive heterogeneity of SCLC contributes to its resistance to current modes of therapy.

1.1.2 Dissecting pathways of SCLC pathogenesis

The neuroendocrine (NE) differentiation phenotype of SCLC coupled with the presence of neurosecretory granules, NE cell and silver absorbing markers initially led to the theory that SCLC originated from amine precursor uptake and decarboxylation (APUD)

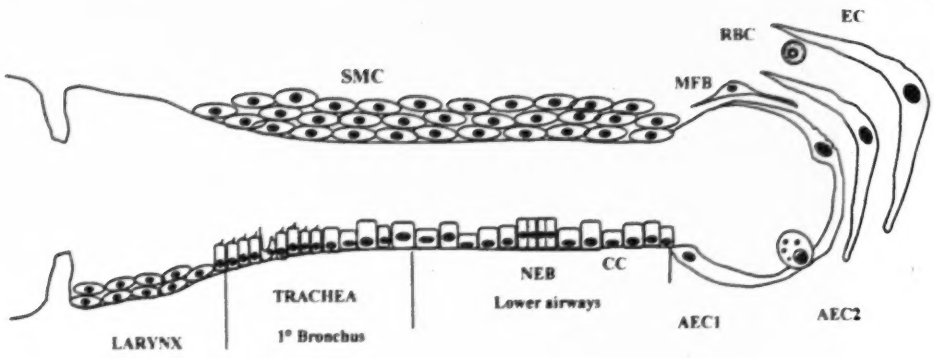
Figure 1. Biological properties of SCLC cells. Classic SCLC cells are relatively small cells, with a high nuclear: cytoplasmic ratio and narrow rims of cytoplasm. They have dense core neurosecretory granules such as **1).** Chromogranin A, which are distinctive features of neuroendocrine (NE) cells. Thus, 'classic' SCLC cells characteristically express high levels of NE markers such as **2).** A variety of opioid and nicotine receptors having characteristics of acetylcholine receptors, e.g. L-dopa decarboxylase (DDC) which can result in the synthesis of dopamine, precursor of norepinephrine and epinephrine; **3).** Neural specific enolase (NSE), **4).** neurofilaments and cell surface antigens such as **5).** Neural cell adhesion molecule (NCAM). **6).** Expression of unique tumor surface antigens may serve as targets for specific SCLC therapy such as monoclonal antibodies alone or conjugated to toxins, radioisotopes or chemotherapeutic agents. Classic SCLC has also been ultrastructurally and biochemically classified as an endocrine tumor belonging to the amine precursor uptake and decarboxylation (APUD) system of cells found throughout the body (149). This diverse group of peptide- or hormone- secreting cells have certain common ultrastructural features such as the high uptake of amine precursors and the ability to decarboxylate them. Embryologically derived from the neural crest, these endocrine cells have been thought to resemble highly modified neurons. Like pulmonary NE cells, APUD cells have granules rich in polypeptide hormones or active amines which can be stained with special histological stains due to their argyrophilic (silver absorbing) or fluorescent properties making them specifically identifiable using light microscopy. **7).** APUD markers such as creatine kinase bovine brain isoenzyme (CK-BB) expression and activity are considered to be specific markers for SCLC. Peptides that have hormonal activity may produce conditions associated with hormone overproduction. **8).** Ectopic ACTH production can be the cause of hypercortisolism. SCLC cells produce important paracrine/autocrine peptide hormone growth factors such as **9).** Bombesin-like /gastrin releasing protein (GRP). **10).** The interaction of GRP with its receptor results in cleavage of membrane phospholipids by phospholipase C to produce inositol phosphates and arachidonic acid, release of calcium from intracellular stores, activation of protein kinase C, and an increase in intracellular pH primarily due to increased activity of the Na+H+ antiporter. These intracellular biochemical changes lead to DNA synthesis and cell proliferation. Other autocrine growth factor mechanisms include: **11).** insulin-like growth factor (IGF-1), **12).** Nerve growth factor (NGF), **13).** arginine vasopressin (AVP) and **14).** transferrin in addition to small polypeptide hormones and other neuropeptides such as bradykinin and neurotensin. **15.)** Underexpression of Class I major histocompatibility antigens and β_2-microglobulin (β_2M) on SCLC may explain in part how these cells evade destruction by normal immunogenic processes. **16).** Deletions in the short arm of chromosome 3 region have been found in 100% of SCLC tumor samples from patients and cultured cells by restriction fragment length polymorphism analysis. Several candidate tumor suppressors that have been cloned and mapped to the 3p chromosome are the β–retinoic acid receptor gene, protein tyrosine phosphatase gamma (PTPγ) and zinc-finger containing genes. Deletions at other chromosomal regions are the retinoblastoma (RB) and p53 genes. Loss of suppressor genes may contribute to permissive expression of the malignant phenotype. **17).** The Myc family of nuclear transcription factors function to control cell proliferation and differentiation. In particular, overexpression of the *myc* oncogene has been correlated with poor prognosis and morphological changes in the SCLC phenotype. *Ras* genes which encode for signal-transducing membrane-associated G proteins are also frequently activated in lung cancers in both early and late stages although activated Ras does not seem to be present in the pre-malignant state.



Adapted from Beck, 1988 (9).

or NE (Kulchitski) cells of the lung (Fig. 1, 1-5; Fig. 2). However, an increasing number of biochemical studies have revealed that considerable heterogeneity exists within a given tumor type. 10% of SCLC cell lines are phenotypically categorized as 'variant' in that they do not express L-dopa decarboxylase (DDC) or gastrin-releasing protein (GRP) (Fig. 1, 2 and 9). Studies of SCLC and NSCLC cell lines have shown the existence of many overlapping characteristics (10). For example, 17% of NSCLC tumors express bombesin reactivity, 50 % have the chromosome 3p deletion associated with SCLC (Fig. 1, 16), as well as expression of DDC, creatine kinase bovine-brain (CK-BB) isoenzyme and other markers previously thought to be specific for SCLC (Fig. 1). Histologically, even at the light microscopic level, cellular heterogeneity exists within individual lung tumors from the same patient. Therefore, it appears that patient tumors contain mixtures of the four lung cancer subtypes and while histological classification is useful, it is limited in terms of defining SCLC pathogenesis. The fact that tumors are of mixed types and that markers are not restricted to SCLC suggests that the observed cell types result from true mixtures within a single tumor. That is, they are heterogenous subpopulations of cells derived from a single tumor cell, and not from distinct cellular origins. With the further understanding of the role of pluripotent stem cell precursors in normal lung tissue function and renewal, it is now thought that lung cancers including SCLC, arise by oncogenic transformation of a neuroendocrine lung stem cell progenitor population (11). The neuroendocrine origin of SCLC and its related stem cell precursor has been highly controversial and has been previously suggested to derive from a distinct neural crest origin in contrast to other bronchial epithelial types. However, the presence of SCLC endocrine markers in NSCLC, as well as the shared expression of epithelial cell markers

Figure 2. Cell types of the mature lung. Depending on anatomic location, the mature airway varies in its composition and structure of cell types from larynx to tracheobronchial to alveolar epithelium. Thus, the larynx is lined with squamous epithelium while the upper tracheal and bronchial airways are lined with ciliated columnar cells, mucous cells and basal cells. Recent experiments by Reynolds et al (17-18) have demonstrated that the NEB microenvironment of the normal and injured lung supports the maintenance of at least two epithelial variants, one with a phenotype intermediate between Clara and pulmonary NE cells and a Clara cell variant. From this evidence, it is likely that pulmonary NE cells serve as a pluripotent stem cell population capable of regenerating Clara cell progenitors similar to that described in the developing lung epithelium.



adapted from Warburton.1998 (13)

Airway lining, secretory and neuroendocrine cells of the lung

Bronchi

numerous secretory cell types such as: mucous, serous, basal (mucosal), intermediate, brush border and Clara cells

Lower airway epithelium

neuroendocrine (NE) cells	argyrophilic staining of pulmonary NE cells has identified three subtypes which may have endocrine, paracrine, receptosecretory, or hypoxia–sensitive functions . The amine and peptide hormones stored in the electron-dense granules of these NE cells likely act on airway smooth muscle and implicate NE cells in epithelial cell growth, branching morphogenesis and carcinogenesis.
Kultschitsky cells	electon-dense, argyrophilic, NE cell which may be an APUD variant
Clara cells (CC)	columnar-shaped secretory cells which are a progenitor of itself and ciliated cells
neuroendocrine or neuroepithelial bodies (NEB)	organoid clusters of Clara cells and NE cells, the NEB microenvironment represents a unique "reserve" progenitor-cell population in the mature lung that participates in airway repair and is capable of restoring the airway epithelium after acute airway injury
alveolar type II epithelial cells (AEC2)	cells with limited progenitor capacity which line the alveolar epithelium, they appear to be able to undergo reversible transdifferentiation to replace type I cells which become lost during injury
alveolar type I epithelial cells (AEC1)	highly specialized cells not capable of multipotent differentiation which also line the alveolar epithelium

EC, endothelial cell; RBC, red blood cell; MFB, myofibroblast; SMC, smooth muscle cell

such as cytokeratin intermediate filaments, implies a common stem cell of endodermal rather than neural crest origin as previously believed (11-13). This evidence suggests that cell type transitions take place that contribute to lung cancer progression and interfere with effective treatment. Thus, lung cancer is now considered to represent a continuum of cell types that utilize differentiation pathways common to normal bronchial epithelium and it is this differentiation capability that gives rise to their capacity to metastasize (11,12).

1.1.3 Molecular origin of SCLC

While SCLC is well defined clinically and morphologically, its histogenesis remains obscure. The events required for lung tumor progression and metastasis are currently being delineated. Unlike colon and breast cancers, there does not appear to be a genetic predisposition to lung cancer, although increased levels and expression of cytochrome-linked enzymes associated with carcinogen metabolism seem to be a risk factor for the disease (3). However, the lung is continually exposed to carcinogens and mutagens and therefore has a high potential for accumulation of genetic insults. Tobacco smoke is one such environmental agent known to directly accelerate the rate of mutations. Thus, the development of lung cancer is associated with multiple genetic events. These genetic changes include activation of proto-oncogenes as well as the inactivation of tumor supressor genes (TSGs). It is estimated that at least 17-22 chromosomal regions with frequent allelic loss are involved, suggesting that inactivation of the same number of putative TSGs are required to transform lung cells (14). Transformation occurs when an appropriate combination of mutations in the cell confers an ability to grow autonomously, invade and metastasize. Only a single mutation is

required for activation of dominant proto-oncogenes. Proto-oncogene products are generally components of signal transduction pathways including growth factors, receptor and non-receptor tyrosine kinases, membrane-associated G proteins such as Ras (Fig. 1,17), cytoplasmic serine/threonine kinases, and nuclear transcription factors such as Myc (Fig. 1,17) (15). Abnormalities in growth factor receptors genes implicated in lung tumorigenesis include: the Her2/neu and epidermal growth factor receptor (EGFR) receptor tyrosine kinases, aberrant expression of the autocrine hepatocyte growth factor / receptor (HGF/ HGF-R) pathway (predominantly in NSCLC pathogenesis) and co-expression of the KIT proto-oncogene (encoding another tyrosine kinase receptor) with its stem cell factor (SCF) ligand. Silencing of TSGs that negatively regulate cell proliferation can also have a substantial effect on tumor cell behavior (Fig. 1, 16). Deletion of both alleles is necessary to inactivate recessive tumor suppressors and loss of the short arm of chromosome 3 is a consistent deletion found in 90% of SCLC and 50% of NSCLC tumors (Fig. 1, 16) (15).

Further evidence for the unicellular origin of lung tumor cells has been provided by DNA fingerprinting methods now available which can determine clonal origin. Thus, lung tumor development and progression occur as result of a dynamic potential for phenotypic transitions via multiple pathways leading to metastatic behavior. Identifying the key events that determine the predominant pathway leading to a particular histological type will be a necessary step in dissecting the pathways of molecular transformation in lung carcinogenesis and metastasis. This will require an understanding of the biology of normal pulmonary epithelial stem cells during lung development and the signaling pathways that regulate their cellular behavior.

1.1.4 Cellular origins of SCLC

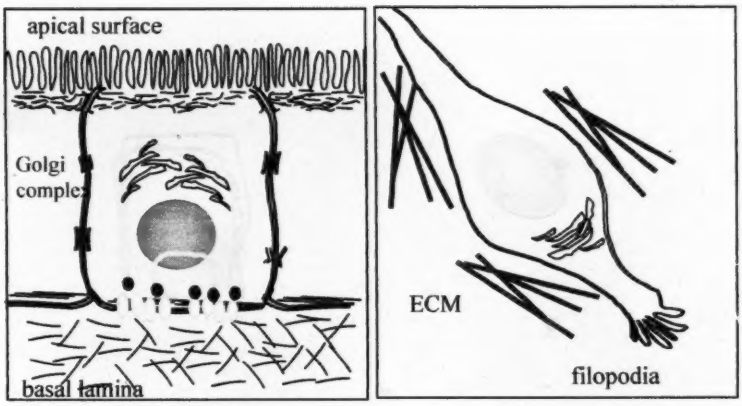
The origin of lung cancer in relation to normal cell types has been highly controversial. The lung is a complex structure utilizing 49 different cell types to form a large diffusible interface capable of exchanging gases to and from the circulation (13). Many of these represent intermediate or differentiating cells belonging to neural or endocrine control systems that can be specific to the lung or similar to cells in other organs (such as APUD cells, Fig.1). The lung develops as a ventral appendage of epithelial cells from the foregut endoderm. The fetal lung then divides laterally into two buds and undergoes cell proliferation, branching morphogenesis and cell lineage differentiation eventually to form the complex respiratory epithelium of the mature lung (Fig. 2). Pulmonary NE and Clara cells have been proposed to be stem cell progenitors involved in epithelial cell renewal. Because they both colocalize to the neuroepithelial body, the progenitor capability of each has been difficult to determine (Fig. 2). Associated changes in the airway epithelial cell composition often involve transient or persistent hyperplasias such as squamous cell, NE cell, basal cell and secretory cell hyperplasia which increase the risk of developing either SCLC or NSCLC lung cancers (12). Unfortunately, the mechanisms involved in the maintenance and activation of airway progenitor cells are still poorly defined. Recent evidence suggests that airway epithelial cells can rapidly change their morphological characteristics both *in vivo* and *in vitro* (16-18). This phenotypic plasticity is consistent with the known variability in tumor phenotypes observed in SCLC (NE-derived) and NSCLC (Clara/ typeII cell-derived) lung

cancers, cell lines and animal model systems. Thus, the airway epithelium is composed of a heterogeneous population of cells that perform a variety of functions some of which have the potential for multiphenotypic differentiation into bronchial epithelial cell lineages depending on the context of their microenvironment (6).

1.1.5 Diversity of lung cell lineage differentiation pathways

Each lung cell type expresses a repertoire of constitutive housekeeping genes expressed by all cells as well as a repertoire of specific genes such as the cytokeratins (CK). Importantly, each cell type also expresses a repertoire of genes specific to its function within the lung. This lung-specific transcription is dependent on complex interactions between several families of transcription factors. The initiation of the program of gene expression by differentiated lung cells results from the inherent properties within each cell and the environmental factors involved in modulating differentiation. In particular, a specific interaction between epithelial cells and surrounding mesenchymal cells is crucial for normal lung branching morphogenesis and cytodifferentiation. These epithelial-mesenchymal interactions are well-coordinated to activate and repress specific transcription factors, peptide hormone and growth factor receptor-mediated signaling pathways, cell cycle control mechanisms, and extracellular matrix (ECM) receptor expression and signaling (Fig. 3). Lung mesenchyme secretes soluble growth factors and hormone mediators into the local environment that stimulate epithelial cell proliferation and lung growth. In addition, growth factor signaling pathways regulate the production of connective tissue components such as collagen, laminin, and fibronectin in the formation of basement membrane by airway epithelial

Figure 3. Epithelial remodeling events in EMT, occurring during embryogenesis and development, implantation, inflammation, wound healing, and lymphocyte trafficking. 1). Changes in cell polarity are associated with loss of apical-basolateral polarization **2).** Changes in spatial cues such as cellular adhesiveness are accompanied by junctional remodeling with loss or changes in cell-cell interactions, and basal bulging **3).** Changes in formation of signaling complexes which relay information to the actin cytoskeleton results in changes in cell shape which are accompanied by elongation of cells and appearance of filipodial protrusions **4).** Localized reorganization of actin assembly and reorganization of the microtubule cytoskeleton leads to apical detachment and redistribution of the membrane trafficking apparatus **5).** Changes in ECM composition, organization, and interactions which correspond with altered integrin adhesion receptor signaling necessary for EMT and migration **6).** Disruption and disorganization of the basal lamina by MMP proteases which degrade matrix proteins **7).** Changes in cytoskeletal organization, distribution of actin and signaling molecules such as vinculin and cell-matrix interactions required for locomotion along migratory paths.



Adapted from Hay, 1995 (20)

specialized forms of ECM deposited as thin sheets that form a continuous barrier and separate epithelial cells from stromal cells

Enzyme-specific degradation of the ECM is under the control of matrix metalloproteinases (MMPs) and their regulators, tissue inhibitors of metalloproteinases (TIMPs)

Adapted from Albelda, 1993

cells (Fig. 3). Interactions between these specific ECM elements and their specific integrin receptor signaling pathways are critical

for dictating cell lineage commitment and geographic restrictions (13). The composition of the basement membrane is determined by the cell-type specific production of ECM molecules and degradative enzymes. Proliferation of airway epithelial cells is accompanied by the release of proteases capable of degrading basement membrane (Fig. 3) (19). This allows newly formed cells to migrate into the space created and the release of connective tissue components and fragments that alter cell proliferation, differentiation and migration. As well, embryonic mesenchymal cells release chemotactic factors that stimulate, direct and regulate the migratory capability of epithelial cells. The degree of complexity in these processes requires precise regulation of epithelial-mesenchymal interactions and maintenance of a balance between matrix production and degradation, which directly influence cellular behavior. As changing environmental interactions are important components of generating tumor cell subpopulations and the appearance of metastases, defining these processes will have implications for both understanding the biology of cancer and designing therapies.

1.1.6 Mesenchymal-epithelial and epithelial-mesenchymal transitions (MET/EMT) in lung development and disease

In early lung development, the process of cell fate determination and transdifferentiation from epithelia to mesenchyme (EMT) and mesenchyme to epithelia (MET) is fundamental for the migration and organizational rearrangement of embryonic cells to form tissues (Fig. 3) (20). This regulated phenotypic modulation involves reorganization of the actin cytoskeleton, and dynamic alterations in cell-cell and cell-

matrix adhesive ability (21). Cell interactions with the ECM contribute to the development of apical-basal cell polarity and this polarization is maintained by both cell-cell and cell-ECM connections to the cytoskeleton. Epithelial-mesenchymal transitions result in a controlled loss of epithelial character leading to decreased intercellular adhesion and cell dissociation. Extended changes in cell architecture, polarity and cell-substratum adhesion are associated with acquisition of a mesenchymal phenotype concurrent with induction of cell motility (22). In the mature lung, similar changes occur in situations of pulmonary injury or hyperoxia. Reversible transdifferentiation into intermediate phenotypes can regenerate tissues in response to cues from ECM, cell shape, and soluble factors in the lung epithelial microenvironment (13,17,18). Damage to airway epithelial cells is common after infection, inhalation of a toxin, or immune-mediated injury, and in chronic airway diseases such as asthma or bronchitis. As lung cells are an expanding population of cells that are capable of conditional renewal, certain stimuli such as injury results in cell proliferation. The type and extent of injury and signals in the local milieu determine whether cell proliferation results in effective repair or fibrotic remodeling. The release of soluble growth factors and loss of contact with neighboring cells can induce quiescent cells to enter the cell cycle concurrent with changes in cell-cell and cell-matrix signaling. After repeated damage to and disruption of the respiratory epithelium, frequent proliferation and EMT/MET events may eventually result in permanently disordered function and the development of cancer. The incidence of unregulated and pathological EMT occuring as a result of this epithelial cell plasticity has been observed to correlate with malignant potential during progression of human carcinomas (12,23). Metastasis is a complex process involving adhesion, de-adhesion,

invasion, and angiogenesis. As tissue integrity is maintained through extensive contacts between cells and signals generated in response to ECM adhesion, the changes in adhesive properties during EMT/MET are regulated at the level of cell surface adhesion receptors. These changes are complex and involve multiple systems of cell adhesion molecules (CAMs) such as cadherin-mediated cell-cell adhesion and integrin-mediated cell-matrix adhesion (Fig. 4) (24-28). CAMs are involved in various homophilic and heterophilic interactions between cells as well as cells and ECM ligands. These interactions mediate recognition and sorting between functional cell types. Dynamic expression of specific levels and combinations of CAMs occurs at particular times and locations during development and tissue regeneration (29). In particular, the tissue-specific combination and temporal pattern of cadherin and integrin expression are key regulators of cellular events critical for tissue formation and morphogenesis, EMT and cell migration. (29,30).

1.2 Cell-cell adhesion complexes

Epithelial cells are linked to each other via various types of cell-cell junctions (Fig. 5) that play crucial roles in the organization and maintenance of normal tissue architecture and function (27,31). Adherens junctions (AJ), or zonula adherens, are membrane-associated specialized junctions characterized as dense cytoplasmic plaques of assembled protein complexes that are structurally linked to the intracellular cytoskeleton (31). At these sites of cell-to-cell adhesion, classical cadherin complexes, in assembly with cytosolic proteins called catenins, are localized to lateral intercellular membrane regions in association with a circumferential belt of actin filaments (32-34). A diversity

Figure 4A. Families of CAM molecules. **1).** The selectin family, involved in leukocyte-endothelium interactions, contain lectin-like domains that recognize and bind cell surface carbohydrate moities in the counter-receptor or ligand; **2).** The immunoglobulin superfamily (IgSF) of cell surface glycoproteins which play roles in cell-cell interactions in the immune system and elsewhere, and participate in both homophilic and heterophilic interactions with other IgSF members, with integrins or with ECM; **3).** The cadherin family of homologous proteins which exhibit homophilic Ca^{2+}-dependent binding and mediate cell-cell adhesion ; and **4).** The integrin family of cell surface adhesion receptors which bind ECM or cellular ligands.

Figure 4B. Structural features of integrin receptors. Integrin heterodimers consist of non-covalently linked α and β subunits which are made up of about 1,000 and about 800 amino acids, respectively. The β chain contains cysteine-rich repeats (*shown in red*) in the extracellular region. The α chain contains several metal-binding sites for divalent cations (M^{++}).Each integrin receptor differs in the specific cation requirements for activity. Generally speaking, Mn^{2+} and to a lesser extent Mg^{2+} stimulate an activated ligand-binding state. The Ca^{2+}-binding site, on the other hand, is located within a region occupied when the integrin is in a conformation inhibitory to ligand binding and therefore $Ca2^{+}$ is associated with an inactive state.

CELL ADHESION MOLECULES

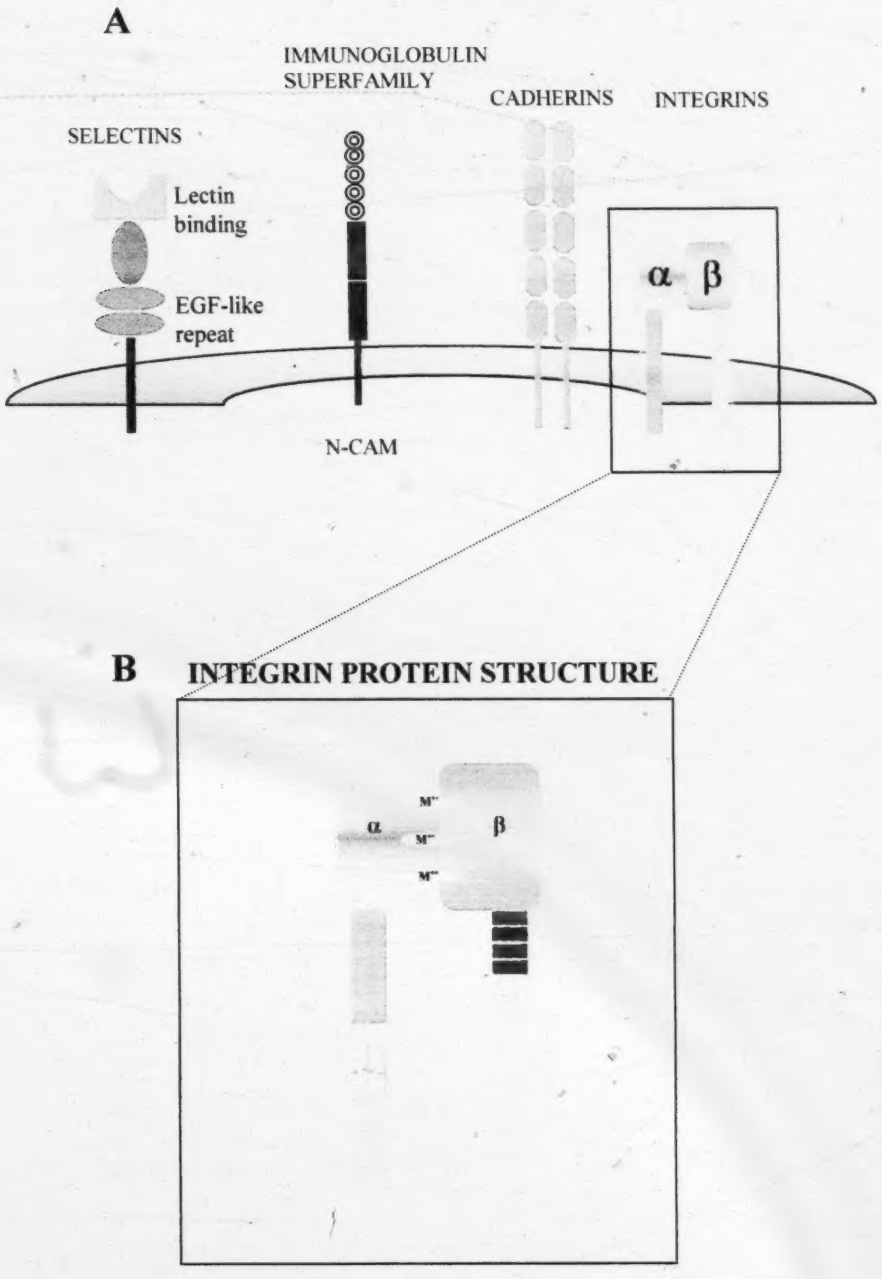
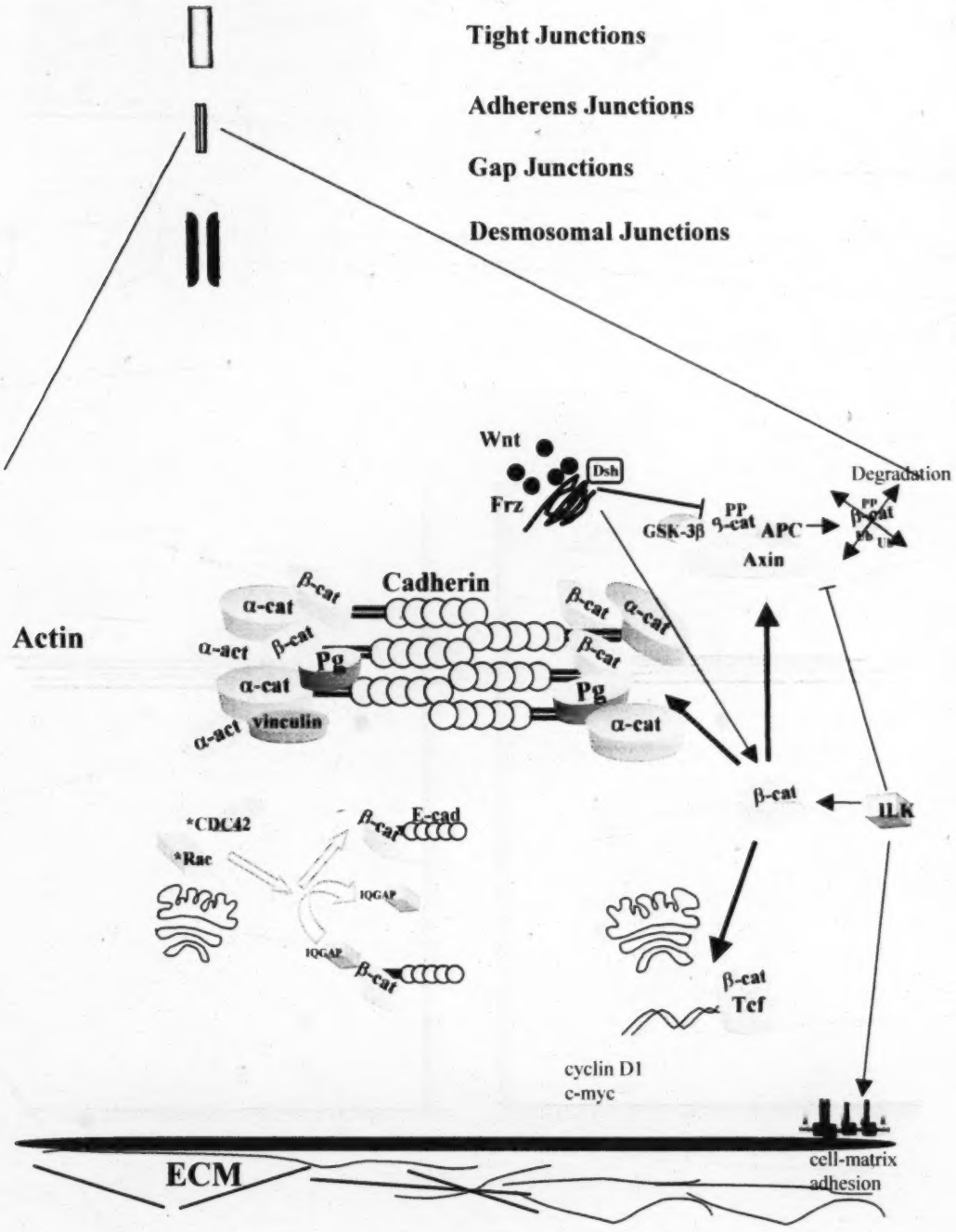


Figure 5. Cell-cell adhesion junctions. β-catenin combines the features of a major structural protein at the adherens junctions (AJ), with that of intracellular signaling in the WNT pathway and transcriptional activation. **1).** Structural role at AJ: linking adhesion receptors of the cadherin family to the actin CSK. **2).** Non-junctional β-catenin is rapidly degraded by the APC-ubiquination-proteosome system. **3).** Plakoglobin, highly homologous to β-catenin and similar in function at the AJ differs from β-catenin in that it is also involved at desmosomes. **4).** Binding of Wnt/Wg ligands to their cell surface receptors of the frizzled family stimulates the activation of kinases and phosphorylation of cytoplasmic proteins, which ultimately leads to changes in the expression of genes involved in cell fate determination and cell proliferation. Activation of β-cat and Pg phosphorylation pathways are controlled by Wnt signals. In the absence of a Wnt-activating signal, non-sequestered free β-cat is rapidly phosphorylated by GSK-3β in the APC-GSK-3β complex and subsequently degraded by the ubiquitin-proteosome pathway. In addition, adaptor proteins such as axin and conductin antagonize Wnt signaling by forming a complex with β-cat and facilitating APC and GSK-3β targeting of β-catenin for degradation (46-47). However, in the event of an activating signal, Wnt binding to its receptor Frizzled (Fzd), the cytoplasmic protein Dishevelled (Dsh) is recruited to the membrane. Dsh binds to axin and inhibits GSK-3β activity. As a result, high levels of β-cat accumulate in the cytoplasm. The increase in the cytoplasmic free pool of β-cat leads to its translocation into the nucleus where it forms a nuclear complex with lymphoid enhancer factor/T cell factor (Lef/Tcf) transcription factors (47,73). β-cat co-activation of Lef/Tcf stimulates the transcription of a variety of target genes. In particular, the transcription and expression of genes specific for proliferation such as cyclin D1 and c-myc, which suppress apoptosis and accelerate cell cycle progression (71-72,74). Interestingly, overexpression of β-cat has also been shown to induce apoptosis, although the mechanism of this is still being determined (75-76). Both β-cat and Pg have a transactivation domain and have been shown to bind Lef/Tcf but the ability of Pg to directly coactivate Lef/Tcf is still unclear (60-61,63). Recently, Pg has been shown to have a direct role in regulating proliferative/apoptotic function in addition to adhesive function, suggesting that it too is a mediator of the Wnt signaling pathway (59,63-64).


Tight Junctions
Adherens Junctions
Gap Junctions
Desmosomal Junctions
Wnt
Dsh
Frz
GSK-3β
PP
β-cat
APC
Axin
Degradation
Actin
Cadherin
α-cat
β-cat
α-act
Pg
vinculin
*CDC42
*Rac
E-cad
IQGAP
ILK
Tcf
cyclin D1
c-myc
ECM
cell-matrix
adhesion

of interacting membrane and PDZ proteins localize at AJs, forming multimolecular complexes, which serve as the framework for the molecular architecture of AJs (35). These proteins network directly with basic AJ components to direct localized actin polymerization and to form the mature AJ in epithelial and non-epithelial cells. Additionally, AJs act as a platform for receiving signals and transmitting them intracellularly. The short-term effect of these signaling complexes is to generate cytoskeletal apical-basolateral cell polarity and transcriptional activation that translates into the long-term maintenance of differentiated epithelial cell and tissue-specific function. For example, aggregation of E-cadherin molecules at the AJ is a primary event that organizes the formation of other cell-cell junctions. Thus, in polarized epithelia, tight junctions (TJs) or zonula occludens, are formed at the most apical part of the lateral membrane. TJs control the selective permeability of cells to ionic diffusion and maintain separation between apical and basolateral membrane compartments. Gap junctions allow the passage of small molecules between adjacent cells. Similar to AJs, desmosomal cadherin adhesion complexes at baso-lateral desmosomal junctions, couple intercellular connections to the cytoskeleton via cytokeratin intermediate filaments. These adhesion structures provide mechanical strength to epithelial cell contacts and are necessary for fully differentiated epithelial cell function.

1.2.1 Cadherin-catenin structural function

Cadherins are morphoregulatory proteins with essential roles in development (27). Loss of function and dominant negative studies have shown that cadherin expression is crucial for establishing initial cell-cell contacts (36). Subsequently, junctions are formed

which change these critical recognition events to long lasting interactions that are necessary for the structure and functional integrity of epithelial tissues (36). Classical cadherins (E-, N- and P-cadherins) are expressed in an epithelial, neural and placental tissue-specific manner, respectively (27). Changes in cadherins during development are associated with transitions between epithelial and mesenchymal phenotypes. For example, changes in expression of N-cadherin (N-cad) in neural crest cells accompany the process of neural crest cell EMT and migration. In this case, N-cad is expressed in neuroepithelial cells but is lost as the cells acquire neural crest mesenchymal cell morphology and neural cell adhesion molecule (N-CAM) monoclonal antibody epitopes (37). With loss of N-cad, N-CAM is expressed on neural crest cells prior to migration but is also gradually lost as migration proceeds. Changes in the levels and types of cadherins expressed by a given cell type are determined by the specificity of function and regulation of adhesiveness required (38). Loss of E-cadherin (E-cad) has a causative role during EMT and in a similar manner in tumor cells, E-cad is frequently lost concomitantly with tumor cell invasion. However, loss of E-cad expression may be associated with the transdifferentiation process and not necessarily the acquisition of invasive capacity (39).

The extracellular regions of classical cadherins consist of five repeating domains that, in the presence of calcium ions, form a rod-like conformation required for adhesive activity (Fig. 4). Cadherins engage in lateral parallel homodimer interactions within the same cell membrane, and form an adhesion 'zipper' with cadherin molecules on opposing cells (Fig. 5) (40). The ability of cadherins to simultaneously self-associate and to link to the actin cytoskeleton enables them to mediate transitions from the initial cell recognition

required for cell sorting of loosely adherent cell clusters to the strong cell-cell adhesion needed for epithelial morphogenesis (41). The structural and adhesive role of cadherins are made possible via intracellular proteins called catenins that link cadherins to the actin cytoskeleton (42). This linkage is required for full-strength cadherin adhesive activity (43). Catenins are cytoplasmic proteins belonging to the Armadillo family (34). Some members of the Armadillo (Arm, the product of the *Drosophila* segment polarity gene) family such as α-catenin (α-cat), β-catenin (β-cat) or plakoglobin/ γ-catenin (Pg) are structural and functional homologues that contain protein interacting domains called Arm repeats (44). Many other Arm-containing proteins, such as the adenomatous polyposis coli (APC) protein, axin/conductin, lymphoid enhancer/ T-cell factor (LEF/TCF) transcription factors and importin among others, can form various interactions via these Arm repeats at different subcellular locations to achieve unique function (45-47). While β-cat associates only with classical cadherins, Pg can form interactions with both classical and desmosomal cadherins. β-cat or Pg form mutually exclusive complexes with classical cadherins. Thus, either β-cat or Pg, bind independently through their N-terminal domain to distal cadherin cytoplasmic domains and to α-cat, which in turn links directly and indirectly to the actin cytoskeleton (Fig. 5) (34). Likewise, at the desmosomal junction, Pg links the desmosomal cadherins to the intermediate cytoskeleton via interaction with cytosolic desmoplakins. Vinculin and α-actinin are additional proteins which can mediate linkage of cadherins to the actin cytoskeleton either directly via β-cat or through binding to α-cat (48,49).

Regulation of cadherin adhesive activity in the adherens junctions is mediated by the cytosolic catenins and their assembly into cadherin-catenin-actin complexes

(26,33,42). Both cadherins and catenins are targets of tyrosine kinases and phosphatases, which regulate the stability of these adhesive complexes via reversible phosphorylation (50). Serine/threonine phosphorylation of the cytoplasmic β-cat binding site in E-cad results in increased binding of β-cat (51). Tyrosine phosphorylation of β-cat, by receptor and non-receptor tyrosine kinases such as EGF-R, MET or Src, results in decreased stability and dissociation of cadherin-catenin complexes. Consequently, β-cat binding to E-cad is downregulated and cell-cell adhesion is decreased (52). Conversely, the continuous activity of phosphatases counteracts the action of tyrosine kinases by maintaining junctional stability. Another member of the Arm family, p120 catenin (p120 cat), was originally identified as a tyrosine phosphorylated protein in Src-transformed cells and in cells stimulated with growth factors (53). p120 cat binds to cadherins at a juxtamembrane site within the cytoplasmic domain and can also link them to the cytoskeleton via α-cat (32). p120 cat appears to have a role in modulating E-cadherin clustering and the initiation of homotypic cadherin adhesion at cell-cell contact sites. Phosphorylation of p120 cat in some transformed cell lines results in destabilized cell-cell adhesion junctions and increased invasion and thus may be a component involved in the cross-talk between cell-cell junctions and the motility of cells (54,55). It is becoming increasingly apparent that α-cat may be more than just a bridge between E-cad-catenin complexes and the actin cytoskeleton but may also serve to organize multiprotein complexes with multiple actin-binding, bundling and polymerization activities (31).

1.2.2 Cadherin-catenin signaling function

In addition to their structural role in stabilizing E-cadherin-mediated adhesion, β-catenin and Pg are involved in signal transduction pathways (56). Such pathways are indispensable in modulation of cell cycle, cytoskeletal remodeling, morphological changes, cell growth, differentiation, motility and apoptosis. β-cat is usually sequestered in E-cad adherens complexes while Pg is additionally found at desmosomal cadherin complexes (45). The amount of cytosolic β-cat and Pg available to participate in adhesive complexes is determined by their interactions with various binding partners involved in different pathways (Fig. 5) (57). A balance between cell adhesion and cell proliferation/differentiation is achieved by regulating the levels and function of β-cat and Pg via adhesive, degradative and nuclear translocation pathways (Fig. 5) (57-59). It is now thought that Pg may have an indirect role in regulating the amount of signaling-active β-cat available in the cytosolic pool as it binds E-cad with lesser affinity than β-cat and interacts with many of the same binding partners (60-64).

The major regulator of cellular levels of β-cat and Pg is the APC protein, which was first identified as the product of a tumor suppressor gene mutated in colon cancer (65). The association of an inherited predisposition to colon carcinomas with loss of function mutations in the APC protein was also correlated to elevated levels of β-cat. Subsequently, APC was shown to be necessary for the rapid degradation of excess β-cat or Pg (66-68). β-cat levels are negatively regulated by formation of a complex between APC-β-cat and the glycogen-synthetase kinase-3β (GSK-3β) which results in phosphorylation of APC and β-cat (69). Serine/threonine phosphorylated β-cat is rapidly

targeted to and degraded by the ubiquitin-proteosome pathway. Mutations in APC or in β-cat itself, which lead to dysregulation of the β-cat signaling pathway are important events in colorectal tumorigenesis (70-72).

Further insight into this pathway was brought about by the identification of β-cat and Pg as critical components of the Wnt-mediated signaling pathway (Fig. 5) (44,45,63) The vertebrate Wnt protein family and its *Drosophila* homologue wingless (Wg) are secreted/soluble glycoproteins involved in signaling pathways which regulate important developmental decisions during differentiation. Not suprisingly, they have also been implicated in contributing to aberrant cell function, for example, in tumorigenesis. Stabilization of β-cat by Wnt signaling leads to accumulation of nuclear β-cat, which complexes with lymphoid enhancer/ T-cell factor (LEF/TCF) transcription factors in the regulation of cell fate (47,73-76).

Simultaneous signaling and convergence of cell-cell, cell-matrix and growth factor pathways enable a cell to integrate multiple positional and biochemical inputs. For example, the serine/threonine integrin-linked kinase (ILK) phosphorylates GSK-3β to inhibit its activity resulting in β-cat translocation into the nucleus (Fig. 5) and is associated with mesenchymal transformation in mammary epithelial cells (77). In addition, ILK acts to downregulate the expression of E-cadherin and upregulate Lef-1 expression (78). Thus, changes in cell-cell and cell-matrix adhesion can modulate gene expression and cell fate. The possibility that cell adhesion-mediated signals govern complete genetic programs, such as EMT during embryonic development or carcinogenesis is becoming increasingly likely (79,80).

1.2.3 Role of small GTPases in maintenance of cadherin-catenin adhesion complexes

The Rho (Ras-homologous) family of small GTPases, which includes Rho, Cdc42 and Rac, are involved in the development and maintenance of epithelial morphology through their essential roles in regulating the actin cytoskeletal dynamics that determine cell shape, growth, polarity and adhesion or migration (81,82). Modulating cadherin-mediated intercellular junctions is one mechanism by which Rho GTPases are currently being shown to regulate cell morphology (41,83-85). Small GTPases have both GDP/GTP-binding and GTPase ability. They cycle between either GDP-bound (inactive) or GTP-bound (active) states, regulated by interaction with specific accessory proteins. Guanine nucleotide exchange factors (GEFs) act to stimulate dissociation of GDP so that the more prevalent GTP can bind. Opposing GTPase activating proteins (GAPs) catalyse the rate of GTP hydrolyis to the GDP-bound form thus leading to small GTPase inactivation. These proteins insure that activation and inactivation of Rho GTPases are tightly regulated, both spatially and temporally, in order to generate specific and localized effects (86). Thus, GTPases are perfectly suited to act as regulatable "molecular switches" via coupling of the active GTP-bound form to specific effector molecules. The effector mechanism by which the Rho family of small GTPases mediate remodelling of the cadherin-catenin complex is just beginning to be characterized (87,88). The small GTPases, Rac and Cdc42, are thought to influence adherens-junction formation through promotion of the assembly of stable E-cad-β-cat-α-cat complexes. One pathway through which this can be achieved is activation of a RasGAP-related protein (IQGAP1), an effector of Cdc42 and Rac (87). IQGAP1 negatively regulates E-cad mediated cell-cell adhesion by directly interacting with β-cat and the cytoplasmic domain of E-cad. As the

IQGAP1-binding domain on β-cat overlaps with that of α-cat-binding domain, β-cat is displaced from binding to α-cat and the E-cadherin-catenin-actin adhesion complex is dissociated. Hence, IQGAP acts as a competitive inhibitor of the functional cad-cat complex formation. Activated Cdc42 and Rac1 positively regulate E-cad mediated cell-cell adhesion by sequestering IQGAP and thereby inhibiting its interaction with β-cat (89,90). Rho GTPase maintenance of cadherin-mediated adhesion may be regulated via Tiam-1 (T-lymphoma invasion and metastasis gene 1). Tiam-1 is a GEF for Rac1 which localizes to cell-cell contacts. Tiam-1 expression has recently been shown to inhibit HGF-induced cell scattering in MDCK2 cells and also to reverse the loss of adhesion exhibited by Ras-transformed MDCK2 cells (91). In that study, expression of Tiam-1 or constitutively active Rac (RacV12) reverted the mesenchymal and invasive phenotype of Ras-transformed MDCK2 cells to a non-invasive epithelial phenotype by increasing E-cadherin-mediated homotypic cell-cell adhesion (91). Thus, localization of Tiam-1 and activation of Rac-mediated F-actin polymerization at adherens junctions may facilitate firm associations between the E-cad complex and the cortical actin cytoskeleton thereby leading to increased cell-cell adhesion (91). P120 cat may also have a role in regulating Rho family GTPase activation/function (92). A mechanism has been proposed by which the activity of Rho GTPases is negatively or positively modulated by the cytosolic and cadherin-associated pool of p120 cat, respectively (88,92). In addition, Ras-mediated small GTPase signaling pathways may also be linked to cadherin-catenin function and the dissociation of the cadherin complex through the Shc adaptor protein. Shc has been recently shown to form a complex with the cytoplasmic domain of cadherin (93,94). Shc can be activated by both growth-factor mediated and integrin-mediated signaling

pathways. Activated Shc recruits the Grb2/Sos exchange factor for Ras small GTPases thereby linking these pathways to the ERK/MAPK mitogenic pathway in the determination of epithelial survival, proliferation or differentiated phenotype. Correct spatial activation of small GTPases are imperative for their ability to both be regulated and to regulate these processes.

1.2.4 Role of cadherin-catenin complexes in EMT and tumorigenesis

As the terminal cytoplasmic components of the Wg/Wnt pathway, it is now clear that catenins are pivotal elements in complex signaling pathways that control cellular motility, growth and differentiation (44). In this regard, cadherins can be connected with other integral membrane proteins such as the EGF-R and ErbB2 receptor that modify cadherin adhesive function to allow cell rounding and membrane ruffling, events important in mitosis and cell migration (33). Migrating cells during development express cadherins in a subpopulation-specific manner: in neural crest cells, the level of α-cat localizing at cadherin contacts between cells increases as these cells differentiate. The increased transcription and upregulation of α-cat in the adherens junctions may be the mechanism by which tighter cell-cell associations are induced and by which these cells cease to migrate (28). The amount of catenins available to participate in assembly of cadherin-catenin-actin complexes is variable depending on the cell type and differentiation status of cells, and is modulated by cellular signals such as those associated with proliferation, polarization of cells and growth factors during development. The E-cad-catenin complex is necessary for development and maintainance of epithelial organization. As morphoregulators, cadherins play critical roles in the

dynamic rearrangement and separation of cell layers and tissues types as well as cell migration that occur during EMT/MET. Dynamic rearrangements of cell-cell adhesion underlie a diverse range of cell processes and are involved in tumor metastasis. Changes in the expression or structure of the cadherins or catenins leads to adherens-junction disassembly, destabilization of cell-cell adhesion and contributes to invasion and metastasis of epithelial tumor cells (23,95). Loss of E-cad-mediated cell-cell adhesion contributes to the transition from benign, non-invasive tumors (adenoma) to malignant invasive tumors (carcinoma) (96). E-cad expression is downregulated in epithelial tumors from various tissues and has been correlated with increased invasiveness of breast cancer (97). Re-expression of E- cadherins in malignant cells has been shown to have a tumor suppressor effect (52). Similarly, invasive, fibroblast-like carcinoma cells can be converted to a noninvasive phenotype by transfection with a cDNA encoding E-cad. Mutations in the cadherin-catenin signaling pathways such as truncation or mutation of β-cat, α-cat, APC or aberrant alterations in β-cat phosphorylation can also contribute to loss of E-cad function (70). In addition, the balance between Rac and Rho activity determines cellular morphology and migratory behavior (98). Dysregulation of cadherin-mediated cell-cell adhesion by Rho GTPases are a likely cause for tumor metastasis and are curently being investigated. Modulation of localization and activity of small GTPase signaling pathways may be one means by which oncogene-, growth factor-and adhesion-induced signaling converge to regulate EMT and the migratory behavior of epithelial (tumor) cells (85,99).

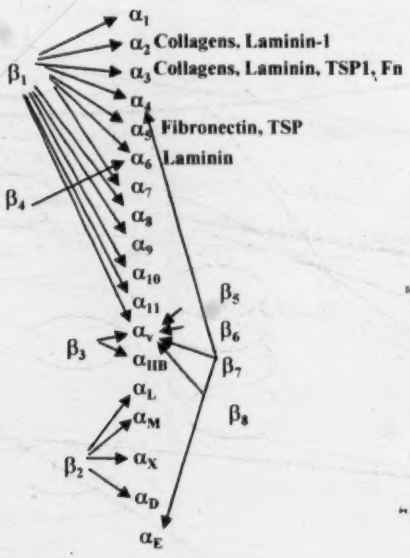
1.3 Cell-matrix adhesion complexes

1.3.1 Integrin adhesion receptors

Cell matrix adhesion is mediated by a variety of cell surface adhesion receptors. Integrins are the primary mediators of cell adhesion to the ECM and are the focus of this thesis. The identification of the integrin superfamily provided a major breakthrough in the understanding of communication between a cell and its ECM environment. Originally characterized by their ability to recognize and bind an Arg-Gly-Asp (RGD) sequence present in many of the ECM proteins, integrins were found to be negatively charged, N-glycosylated type-I-transmembrane glycoproteins (100). Subsequently, it was shown that integrins can also bind to cell surface ligands and are involved in mediating certain cell-cell interactions, particularly in the cells of the immune system (101). Integrin structure has been reviewed extensively (102,103). Briefly, integrins have a large globular N-terminal extracellular domain, a transmembrane segment and short C-terminal cytoplasmic domains of less than 60 amino acids (Fig. 4B). To date, the only exception to this is the β4 subunit, an integrin involved in hemidesmosome assembly, which has over 1000 amino acid residues in an extended cytoplasmic domain. Integrin receptors consist of noncovalently linked α and β -subunits (Fig. 4B). So far, 18 α-chains have been identified which can associate with at least 8 β-chains in various combinations to form 24 different functional receptors. The specific combinations of α and β -subunits and associations in various adhesion complexes, determines the molecular specificity of the integrin receptor for ligand-binding (Fig. 6A) (104). Cell interactions with the ECM are complicated by the fact that integrins display functional redundancy. That is, the same integrin heterodimer can recognize different ECM proteins, and one ECM component can

Figure 6A. Integrin subunits and their ECM ligands. Distinct integrin α/β heterodimers exhibit specific and often overlapping ligand binding capacity to the various ECM proteins. For example, $\alpha_6\beta_1$ is a laminin receptor, whereas $\alpha_3\beta_1$ binds to laminin-1 and 5 as well as collagen, thrombospondin 1 (TSP1) and fibronectin. Four integrins, namely $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$, are collagen receptors but vary in their ability to recognize distinct collagen subtypes as well as signaling function. Although in theory any of the α / β associations should be possible, in practice integrin combinations expressed in vivo can be classified into three subfamilies, each characterized by a common β subunit bound to distinct α subunits. β_1 and β_3 integrin subsets are involved primarily in interactions with ECM proteins, with the β_3 subfamily directed at vascular ligands such as von Willebrand factor (vWf), fibrinogen and thrombospondin. The β_2 subfamily is restricted to leukocytes and their interactions with immunoglobulin superfamily (IgSF) ligands on opposing cells. β_2 integrins are central to lymphocyte homing, migration of leukocytes at sites of inflammation, and cell-cell interactions that lead to immune responses. **B. Integrin β-propeller domains.** Domains homologous to the integrin β-propeller domains have been found in enzymatic and non-enzymatic proteins such as phosphatidylinositol phospholipase D (PIPLD) and GTP-binding proteins suggestive of a functionally significant evolutionary conservation (254). Thus, a model has been proposed in which integrin ligands and a Mg^{2+} ion are predicted to bind to the upper face of the β-propeller in a manner analagous to the substrate-binding face of β-propeller enzymes and that used by the α and β subunits of GTP-binding proteins. The I-domain of the α-subunit sits on top of the β-propeller domain connected by a hinge region, which allows movement of the domains relative to one another. Like the switch II region in the G protein, the integrin β-propeller fold may function as an affinity switch leading to conformational changes between the subunits. **C. Structural features of the tetraspanin superfamily.** Tetraspanins have short intracellular N- and C-termini domains, and two highly divergent hydrophilic extracellular loops that vary considerably in sequence and length between different tetraspanin proteins, although they contain four highly conserved cysteines (177). Currently, only the crystal structure for the large extracelluar domain of human CD81 has been determined. In addition, tetraspanins share four highly evolutionarily conserved membrane-spanning hydrophobic domains. A CCG motif in which the position of the last cysteine is conserved in all tetraspanins (*shown in pink*); a conserved PxxCC motif is found in >65% of tetraspanins (*shown in blue*), in the second extracellular loop. Consensus sites for N-linked glycosylation in the large extracellular loop, found in most tetraspanins (except CD81 and NET-2), are indicated by a black circle.

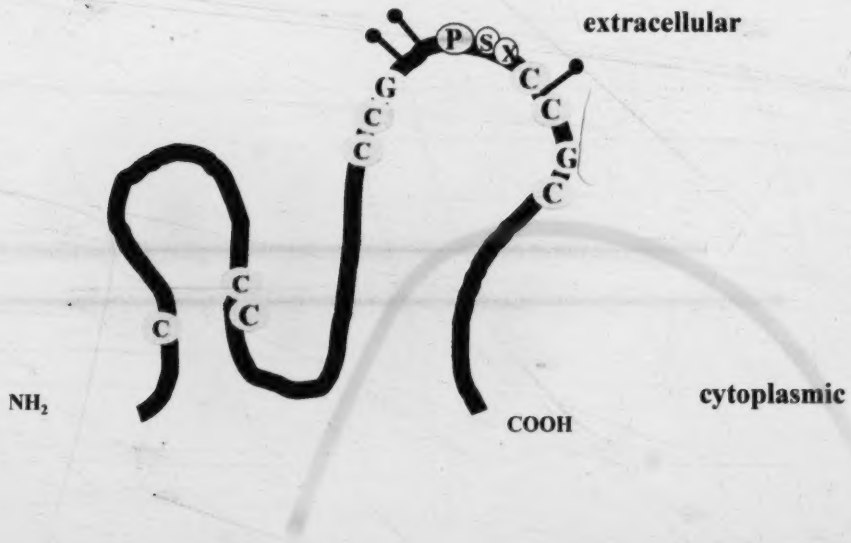
A



B INTEGRIN PROPELLER DOMAIN

adapted from Fernandez, 1998 (108)

C TETRASPANIN PROTEIN STRUCTURE



be recognized by multiple integrins (Fig. 6A). Thus, the functional status of ligand binding and regulation of integrin ligand-binding activity seems to be exceedingly complex. Extracellular ligands can exist in several specific isoforms and can be multivalent, having multiple conformation-dependent and independent recognition sites in one ligand (105,106). Additional integrin versatility is achieved by the fact that many of the cytoplasmic domains can be alternatively spliced to create different isoforms. Thus, despite what appears to be a functional redundancy, signaling differences between integrin subsets are facilitated by specific α / β integrin heterodimer combinations, splice variants and via association of function-specific regulatory proteins.

Tertiary structure models have provided added insight into how integrins achieve these signaling differences through the organization of their ligand recognition sites. The extracellular N-terminal half of all integrin α–subunits contain homologous repeats that are predicted to fold into a β–propeller domain containing seven four-stranded β–sheet blades arranged in a cyclic ring structure (Fig. 6B) (107). Folding of this three-dimensional structure is dependent on correct α and β subunit association, forming a ligand-specific globular head domain allowing conformational binding of specific ECM components. The presence of divalent cations bound within the cation binding sites induces a conformational relaxation in the integrin resulting in exposure of ligand-binding sites, thought to lie near an interface between the two subunits. Like the switch II region in the G proteins, which contain β-propeller domains homologous to integrins, the integrin β-propeller fold may function as an affinity switch leading to conformational changes between the subunits (108).

1.3.2 Integrin activation

The investigation of structural requirements of membrane-proximal and extracellular domains involved in ligand recognition, ligand binding and specificity of heterodimer formation has provided insight into understanding the mechanisms of integrin function and bidirectional ability to transduce signals (108-110). It is believed that integrin extracellular, transmembrane and membrane-proximal domains form a "hinge" in which ligand-binding signals can be generated extracellularly to change intracellular conformations (outside-in signaling) and release the inactivating constraints of the α subunit on the β subunit (111). Alternatively, intracellular signals generated via associated integrin regulatory proteins can disrupt this interaction, initiating conformational alterations that are transmitted across the transmembrane to the extracellular domain. This induces exposure of extracellular ligand-binding sites (inside-out signaling), modulating the affinity of the integrin for ligand binding and resulting in integrin activation (112). Integrins, therefore, are bidirectional signaling molecules, whose function is responsive to, and regulated by, both extracellular and intracellular environments (113).

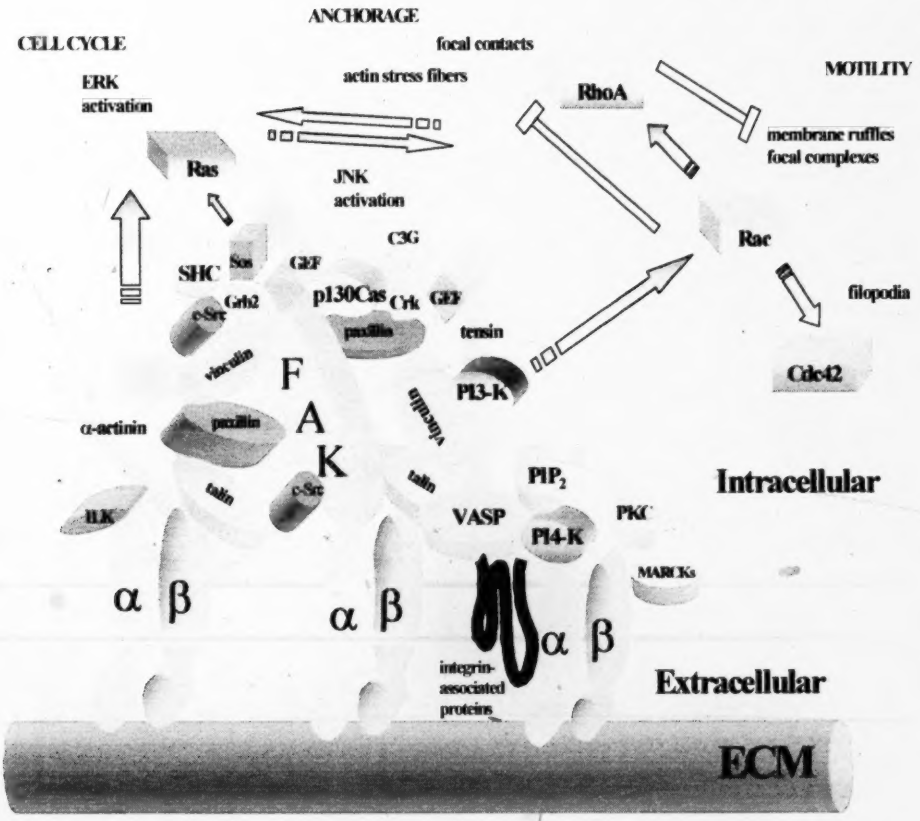
A proposed "allosteric" model of integrin activation, comparing integrin regulation to the behavior of allosteric proteins, predicts that integrin ligand-binding can exist in three conformational states (114,115). One, an inactive state in which α subunit structural constraints maintain the integrin in a default "off" position. Two, a ligand-induced active state, in which intermediate, transient and low affinity binding to the ligand alters the conformational state of the integrin. This causes increased exposure of the ligand-binding site to additional regulation, and allows a third, high-affinity, ligand-

occupied state in which integrin-ligand interactions and therefore adhesion is stabilized. Conformational transitions in integrin activation state induces the appearance of novel epitopes that can be detected by activation-specific mAbs (116). Divalent cations are essential for integrin activity and can alter both integrin affinity and specificity for ligands.

1.3.3 Ligand binding affinity and avidity

The adhesiveness of an integrin receptor for its ligand can be increased in two ways. One is by the induction of a high affinity state. Both changes in inside-out or outside-in signaling can alter conformation-dependent integrin affinity and therefore activation of an integrin for ligand-binding. The other mechanism is through the engagement of several low affinity receptors clustered together to increase the overall binding strength in a localized area thus creating high density and high valency integrin complexes that have increased avidity for ligands (117). The degree of integrin activation, state of cell adhesiveness required and bidirectional signal transduction within a cell is situational. In particular, integrin activity can be defined by differences in cell type and by the differentiation state of the cell (55,109). As suggested, the state of cell adhesiveness is not regulated solely by the state of integrin activation or, ligand occupancy (118). Initiation of signaling and thus stable adhesion requires integrin clustering and formation of multimolecular complexes with other proteins (Fig. 7) (117). Consequently, a combination of, or equilibrium between, modulation of avidity with that of affinity, may be required for successful adhesion to ligands (119). The relative contributions of affinity and avidity to ligand binding would be expected to vary with

Figure 7. Schematic diagram of integrin-associated complexes. Protein-protein and protein-lipid interactions regulate the assembly of multimolecular complexes and the subcellular location of focal adhesion proteins. **1).** SH3 domains bind to proline-rich sites and are critical for coupling to effector molecules and targeting of signaling complexes to discrete sites within the cell. **2).** Tyrosine phosphorylated residues are binding sites for SH2 domains found in many proteins,adaptors and enzymes. PTB domains recognize motifs that contain a phosphorylated tyrosine residue. Many focal adhesion components are phosphorylated, for example, paxillin, tensin, talin, vinculin, the ERM family and zyxin. These are regulated by enzymes such as serine/threonine (ILK) and tyrosine (FAK, c-Src) family kinases, and tyrosine phosphatases. **3).** A large number of adaptor proteins (p130Cas,Shc,Grb2,Crk) mediate interactions between actin-bound or integrin-bound components, enzymes, and GEFs for small GTPases (Sos, C3G). **4).** Actin-binding proteins such as talin, α-actinin, tensin, filamin and ERM proteins are a direct link between integrins and the cytoskeleton. Actin-regulatory proteins such as those involved in actin polymerization (profilin, ARP 2/3 complex), depolymerization (ADF/cofilin), severing/capping (gelsolin), and cross-linking (talin, filamin, vinculin, α-actinin) modify localized actin cytoskeletal dynamics. Many of these cytoskeletal proteins can bind both focal adhesion components and acidic phospholipids, membranes and actin. **5).** The membrane phospholipid, phosphatidylinositol, can be phosphorylated either singly or in combination by various PI kinases to generate polyphosphorylated inositol lipids or phosphoinositides (PIs), which act as important signaling intermediates. Proteins containing PH domains can interact directly with the cell membrane by binding to PIs. The control of local concentrations of these actin-binding proteins are regulated by phospholipids and phosphoinositides by associated regulatory molecules such as MARCKS (myristoylated alanine-rich C-kinase substrate), VASP and $PI(4,5)P_2$.


ANCHORAGE
CELL CYCLE
focal contacts
actin stress fibers
MOTILITY
ERK
activation
RhoA
membrane ruffles
focal complexes
Ras
JNK
activation
C3G
Rac
SHC
Sos
GEF
filopodia
Grb2
p130Cas
Crk
GEF
c-Src
paxillin
tensin
vinculin
F
Cdc42
PI3-K
vinculin
α-actinin
paxillin
A
K
talin
talin
PIP2
Intracellular
ILK
c-Src
VASP
PKC
PI4-K
MARCKs
α β
α β
α β
integrin-
associated
proteins
Extracellular
ECM

type of integrin, cell-type specific intracellular factors, and the context of the cellular microenvironment (120). Integrin activation and subsequent assembly of specific molecular complexes with cytoskeletal and signaling elements determines the fate of cells in response to microenvironmental cues (102).

1.3.4 Integrin function

1.3.4.1 Assembly of cytoskeletal and signaling complexes

As transmembrane connectors, integrins engage the ECM through their extracellular domains and interact with cytoskeletal elements via their cytoplasmic domains (Fig. 7). Inside-out signaling, as a result of extracellular stimuli such as soluble growth factor or cytokine signaling, involves cytoplasmic regulatory proteins that bind the α subunit and affect the activation state of the integrin and therefore its affinity for ligand (120). Thus, the α chain cytoplasmic domain seems to specify the function of the integrin and regulate its ligand affinity. Sequences in the cytoplasmic domain of the α subunit appear to be responsible for determining the specificity of integrin location, by restricting the access of β tail-binding proteins until integrin activation (121). Integrin activation requires both changes in affinity and in lateral mobility of integrins in the membrane. Ligand binding, integrin clustering, and outside-in signaling cause a release in the inhibitory constraints of the α subunit cytoplasmic domains on the β domains. Consequently, default sites in the β cytoplasmic domain become exposed to binding of cytoskeletal and signaling molecules (122). Integrin release from inhibitory cytoskeletal linkages and localization to specialized adhesion structures at discrete sites of cell-ECM contact subsequently results in anchoring of the cell to the cytoskeleton (123). At these

sites of focal contact, integrin complexes with specific proteins are assembled (Fig. 7). Formation of focal complexes results in local recruitment and organization of actin fibers, association of integrins with the actin cytoskeleton, and subsequent activation of multiple signaling cascades (113,124). Using interference reflection microscopy, these can be visualized *in vitro* as focal adhesions: dense cytoplasmic plaques formed at points of close contact between the plasma membrane of adherent cells and immobilized substrate of the tissue culture dish (125). More specifically, focal adhesions correspond to the ends of tightly packed bundles of actin microfilament stress fibers and highly stabilized adhesion as a result of cell spreading on a rigid surface. Because these *in vitro* focal adhesions are representative of focal contacts found *in vivo* they have been used to study and dissect the mechanisms of integrin assembly and signaling pathways.

Integrin subunits otherwise have no intrinsic enzymatic activity although tyrosine phosphorylation of β subunit cytoplasmic NPXY sequences has been found to occur which may regulate cytoskeletal or protein-protein interactions. Thus, directly-or indirectly-bound intracellular proteins are essential for connection of integrins to effector signaling pathways. Sequence-specific motifs in the β subunit, in particular NPXY motifs, display direct, specific and often overlapping binding sites for recruitment of structural and signaling/regulatory cytoskeletal proteins such as talin, filamin, tensin, and α-actinin (Fig. 7). Recruitment of adaptor proteins such as paxillin, Shc and Grb2, and regulatory intracellular protein kinases link integrin activity with intracellular pathways throughout the cell and define its morphology and behavior.

1.3.4.2 Signal transduction function of integrins

Integrin-mediated cell adhesion triggers a variety of metabolic events such as calcium influx, K^+ channel activation, small GTPase activation, inositol lipid metabolism (phosphatidylinositol-4,5-biphosphate or PIP_2 synthesis), and activation of tyrosine and serine/threonine kinase phosphorylation cascades (111). Initial analysis of signaling events downstream of integrin-mediated cell adhesion identified a major phosphorylated protein of 110kd with tyrosine kinase activity known as $pp125^{FAK}$ or focal adhesion kinase (FAK) (126). FAK localization to focal contacts can be mediated indirectly via its C-terminal binding sites for talin and paxillin, or directly via binding to the cytoplasmic domain of the integrin β subunit. Ligand-dependent integrin aggregation and oligomerization causes activation and autophosphorylation of FAK (111,117). Autophosphorylation of FAK at a critical Y397 residue, serves as the binding site for the SH2 domain of c-Src or c-Fyn family of protein tyrosine kinases (127,128). Src binding is crucial for further phosphorylation of FAK at Y925 as well as phosphorylation of FAK-associated proteins (128). The FAK-Src kinase complex regulates the assembly and disassembly of focal adhesions during cell migration and protection from cell death mechanisms (129-131). Activation of Src kinases results in phosphorylation of associated adaptor molecules such as Shc (132). Phosphorylated Shc recruits Grb2/Sos, the guanine exchange factor for Ras, to the plasma membrane thereby promoting activation of the Ras-extracellular signal-regulated kinase (ERK) pathway (Fig. 7) (132,133). FAK is a pivotal regulator linking integrin signaling to pathways controlling cell growth, death and survival (126). Not only are there multiple pathways from FAK to ERK activation, but many interconnecting pathways are generated via activation of a multitude of signaling

intermediates. Consequently, integrin signal transduction has been discovered to involve complex networks of spatially and temporally organized interconnecting signaling pathways, and has generated a vast amount of literature (134).

1.3.4.3 Structural role in cell adhesion

Integrins have a structural role in mediating physical attachment of the cell to the underlying ECM. In addition, integrin attachments have an immediate effect on cell shape by exerting physical forces on the nucleus, which provides the cell with structural and mechanical information (135-137). As mechanotransducers, therefore, integrin attachments directly regulate gene expression, independent of any biochemical signaling pathways (138,139). Thus, both the strength of integrin-ligand binding (activation) and the degree of attachment or avidity determines: 1) whether the cell undergoes cytoskeletal re-organization in preparation for stabilized spreading and adhesion; 2) whether there is a change in cell shape and rounding in preparation for mitosis; or 3) whether the cell acquires optimal adhesion for traction and the predominance of a leading edge required for cell migration (140). Accordingly, focal complexes are highly dynamic and transient structures with heterogeneous composition that have the potential to mature into different types of focal contacts depending on cell type-specific function (55,139,141).

1.3.4.4 Role of integrins in cell migration and invasion

Cell migration is an intricately complex and coordinated process involving decreased cell-cell adhesion, changes in the cytoskeleton and cell-substratum adhesions, as well as matrix remodeling (55,142). A central question is, how, in response to

microenvironmental cues, does the cell mobilize itself for the changes in cell behavior associated with locomotion? Cell motility can be summarized temporally into several steps: 1) formation of thin, flat membrane extensions at the cell periphery called lamellipodia, which contain a branching network of actin filaments 2) formation of new adhesions 3) cell body contraction and 4) tail detachment (142,143). Regulated adhesion is a prerequisite for cell migration (55). An optimum strength of adhesiveness and therefore integrin-ligand affinity is required, to provide enough tension to generate the contraction force needed for a cell to pull itself across its substrate. Adhesion events at the leading edge of the cell must be coordinated with de-adhesion events at the rear, in order for a cell to move forward (144). These mechanisms are cell type-specific, varying with migratory and invasive capacity needed for cell function.

The order of events involved in the transition from a stationary to an invasive phenotype is slowly being delineated. In epithelial cells, apical-basolateral polarity is disassembled and, as a result of cytoskeletal reorganization, cells acquire a polarized morphology compatible with the development of a leading edge and the directed protrusion of lamellopodia seen in motile cells (Fig. 3). Cell migration is a necessary prerequisite for invasion. A cell must break its contact with the substratum and make new contacts compatible with locomotion. The control of timing, speed, direction, and targeting of migrating cells towards specific ECM proteins (haptotaxis) and diffusible stimulants/ growth factors (chemotaxis) is precisely coordinated and intricately regulated (145). Certain integrins, such as $\alpha_V\beta_3$, $\alpha_3\beta_1$ and $\alpha_7\beta_1$, are associated with cell motility more than other integrins (145). Although $\alpha_6\beta_4$ mediates strong adhesion through hemidesmosome formation, it has a highly invasive capacity when present in certain

carcinomas (146,147). Once the motility machinery is assembled, cells require the production of ECM degrading and remodelling enzymes in order to be able to move through tissues in response to chemotactic and haptotactic stimuli (148). These proteolytic enzymes are tightly regulated in normal cells (149).

The mechanisms of β1 integrin activation and deactivation underlying migratory and invasive events are still incompletely understood and intertwined with activation of growth factor signaling pathways (150). The extent of integrin activation or deactivation determines the extent of cell-substratum adhesiveness and thus the rate at which a cell migrates (140,151). Initially because of its role in assembly of focal adhesions and formation of stress fibers, the function of FAK was thought to be that of promoting strong cell-matrix interactions. Unexpectedly, studies of the FAK-/- mouse fibroblast cells showed an increase in stable focal adhesions and a drastic decrease in cell migration rates (131). Suprisingly this indicated that rather than simply maintaining the stability of focal adhesions and cell spreading, a more accurate function of FAK may be to initiate signaling pathways leading to increased turnover of focal adhesions. Indeed, activation and autophosphorylation of FAK has been linked to cell migration signaling pathways in response to migratory stimuli such as activated growth factor- and/or integrin- receptors (129,152-154). In addition to FAK activation, integrin regulation of cell migration pathways is also mediated by integrin activation of dual protein/phospholipid kinases such as PI3K (phosphatidylinositol 3-OH kinase) or integral serine/threonine kinases such as members of the protein kinase C (PKC) family (137,155,156). Integrin activation of both PI3K and PKC leads to activation of the Rho family of small GTPases, which regulate actin cytoskeletal dynamics and are required for the changes in cell shape

necessary for cell attachment, spreading, and/or stabilized adhesion versus cell movement (137,143). The activity of kinases such as FAK, PI3K and PKC and their detailed connections to pathways mediating downstream integrin proliferation, survival or migratory signaling function is still unclear (134). What is immediately apparent is the complexity of integrin-mediated kinase activation in defining cell-type specific function, and the coordinate regulation of their activity with that of small GTPases (137,157,158).

1.3.4.5 Role of Rho small GTPases in mediating integrin function

The activity of the Rho family of small GTPases is modulated in response to integrin-mediated adhesion. Whether activation of Rho family members is upstream or downstream of integrins is still unclear, not to mention confusing. The answer is likely to be both, and that it is the multidirectional nature of the relationships between the two that contribute to their complexity (139). What is known is that activation of Rho family members induces cytoskeletal reorganization and clustering of ligand-occupied integrins into multimolecular complexes with cytoskeletal and signaling proteins (159). The role of Rho, Rac and Cdc42 members of the Rho GTPase family in regulating the formation of actin-containing structures has been reviewed extensively (81,86,160). After integrin ligation and cell adhesion to ECM, rapid activation of Cdc42 and Rac contribute to cell spreading (113) (55,161). In general, Rho is involved in the development of focal adhesions, membrane ruffles and in particular, stress fibers; Cdc42 induces filopodia; and Rac induces membrane ruffling and cell motility (86). As mentioned in section 1.2.3, other functions of the Rho family proteins include regulation of cell-cell adhesion, cell proliferation and membrane trafficking (41,162). During epithelial-mesenchymal

transformation, actin-based cytoskeletal reorganization by the Rho subfamily is responsible for loss of apico-basal polarity and gain of motility (163,164). The activity of the Rho family in determining cellular morphology is cell-type and function-dependent (82). Several effector pathways involving Rho-kinase (ROCK), LIM-kinase, p21-activated kinase (PAK) and the ezrin-radixin-moesin (ERM) family have now been characterized (143).

Growth factor and G-protein coupled receptors as well as integrins activate the Rho family of small GTPases (165). The mechanisms by which these small GTPases are activated are not fully determined but may be through activation of kinases such as PI3-K (143,166). The generation of phosphoinositide signaling intermediates by PI3K from membrane phospholipids may recruit GEFs (via lipid-binding pleckstrin homology or PH domains) for these Rho GTPases thereby leading to their activation (167). Products of PI3-K, such as PIP3, may also regulate GEF activity by enhancing its tyrosine phosphorylation thereby further affecting the localization of Rho GEFs not only through the PH domain but also its recruitment by adaptor proteins for phosphorylated tyrosine residues. The changes that occur in cell shape as a result of Rho GTPase activation and cytoskeletal reorganization are important for the direct assembly of focal adhesion complexes and control of cell motility (55).

1.3.5 Regulation of integrin function

1.3.5.1 Integrin-associated proteins

Integrin activation is directly regulated by intracellular factors that bind to integrin cytoplasmic domains (123). As already described, complex cytoskeletal interactions may restrict diffusion of integrins. Ras proteins have been shown to have an

effect on integrin affinity: H-ras being a negative inhibitor of integrin activation whereas R-ras appears to have a positive role in activation of integrins (112). Other intracellular signaling proteins such as cytohesin-1, a GEF for the ADP-ribosylation factor (Arf) family of small GTP-binding proteins; β_3-endonexin; integrin cytoplasmic-domain-associated-1 (ICAP-1); and receptor for activated protein kinase C (Rack1), have been shown to interact with the membrane proximal regions of the various β subunits (123). Prior to heterodimer assembly, integrin α and β subunits can bind chaperone proteins such as calnexin and calreticulin in the endoplasmic reticulum (ER) although the regulation and significance of these mechanisms are still poorly understood (168,169). In addition to cytoplasmic domain associations, integrin function is also modulated by lateral interactions with transmembrane proteins (170). Caveolin is a membrane protein found constitutively in association with β_1 or β_2 integrins that acts as an adaptor protein linking integrin activation to Shc-signaling growth regulation pathways (171). Transmembrane proteins shown to directly associate with β_1, β_2 and β_3 integrins, and other cell surface associations for which direct interactions are yet to be established, are reviewed in (123).

1.3.5.2 Tetraspanin superfamily of integrin-associated proteins

Tetraspanins are a recently discovered family of homologous glycoproteins which directly associate with integrins although their role in regulation of integrin function is still poorly understood (172). Tetraspanins, also known as transmembrane 4 (TM4) superfamily proteins, share sequence and structural homology of four highly conserved hydrophobic membrane-spanning domains (Fig. 6C). While, in humans, some

tetraspanins such as CD9, CD63, CD81 and CD82 are found on virtually all tissues, others such as CD53 (lymphoid) and CD37 (mature B cells) have a more restricted tissue-specific distribution (172). The tetraspanin family has 26 members in humans so far, and is encoded by 35 genes in *Drosophila* (173). The ubiquitous presence of tetraspanins in nearly all mammalian cell types and their expression in evolutionarily diverse species such as *Caenorhabiditis elegans*, *Schistosoma* and *Drosophila* suggest they may play a fundamental role in cellular function. Indeed, tetraspanins have been implicated in a number of diverse cellular processes such as cell activation, co-stimulation, proliferation, motility, adhesion, signal transduction and differentiation (174,175). In addition, tetraspanins regulate morphogenetic functions in cell development, in particular, neural cell adhesion, motility and growth regulation (176). For example, in *Drosophila*, the tetraspanin late-bloomer gene plays a role in the formation of neuromuscular junctions (177). The tetraspanin CD9 is transiently expressed in the development of both central and peripheral nervous system and on B-cells only at certain stages of differentiation (176).

Not suprisingly, tetraspanins have been shown to have a role in suppression of metastasis (178). Expression of tetraspanins has been found to be down-regulated in various human cancers and their expression is inversely correlated with patient survival rate (172,179-183). CD63 (ME491) surface antigen expression, originally a marker for early stages of melanocytic tumor progression, is also expressed in nevocellular nevi, neuroendocrine tumors and adenocarcinomas (184,185). CD63 is not expressed in normal melanocytes and its expression is stage-specific; high in radial growth-phase primary melanomas and low or absent in vertical growth-phase and metastatic melanomas (186-

188). Subsequently, expression of CD63 in NIH-3T3 cells and transfection into human melanoma cells was associated with suppression of growth and metastasis in nude mice (189). CD9 expression is also inversely correlated with the metastatic potential of melanoma (190). In NSCLC, CD9 gene expression is associated with higher survival rate (191). Similarly, decreased CD82 expression in NSCLC may also be involved in malignant tumor progression (192). CD82 expression in cell lines derived from prostate cancers is reduced and its introduction into a rat prostate cancer cell line suppressed experimental metastasis (193). In contrast to these findings, expression of CD82 in human lung cancer cells and its i.v.-injection into NK-depleted SCID mice were found to promote multi-organ metastasis (194). Similar treatment of the human epidermoid carcinoma cell line, HEp-3 with a monoclonal antibody against CD151 (PETA3) specifically inhibited in vivo metastasis (195). In that study, CD151 was linked as a positive effector of metastasis possibly through its ability to mediate cell migration. The finding that tetraspanins are positive and negative regulators of cell proliferation and motility points to a complexity of function equivalent to that of the integrins. Like integrins, tetraspanins are found localized both at the cell surface and within intracellular compartments, and have both cell-matrix and cell-cell distribution (173). While their exact biochemical function is as yet unknown, their association with β_1 integrins and other integral membrane proteins on the surface of diverse cell types have led to the proposal that tetraspanins have a 'molecular facilitator' function (172,178). In this capacity, they may serve to organize the assembly of multimolecular membrane signaling complexes that control cell differentiation, adhesion and motility (196).

1.4 Rationale and objectives

Molecular interactions between tumor cells and ECM can influence their capacity to disseminate (197). However, the functional relationships in adhesive signaling pathways involved in reversible transdifferentiation and phenotypic commitment of pulmonary epithelial cells is poorly understood. Here we present an experimental model system in which a metastatic parental SCLC line and its differentiated derivative with identical genetic content, exhibit phenotypically distinct adhesive states. The development of a phenotypic conversion from spherical tightly aggregated clusters of the non-adherent SCLC parental NCI-H69 cells (designated H69C) to an adherent monolayer (H69B) was experimentally induced by treatment with the differentiating agent, bromodeoxyuridine. This phenotypic conversion was associated with reduced *in vivo* tumorigenicity when the derived cells were injected into severe-combined immunodeficient (SCID) mice. Therefore, the objectives of this study were:

1) To confirm the genetic identity of H69C and H69B cells.

2) To characterize the changes in cytoskeletal and cell adhesion proteins during the morphological transdifferentiation of H69C to H69B cells.

3). To characterize the differences between the two cell types in expression, profile and function of integrins and their associated tetraspanins.

and 4). to determine whether the differences exhibited in cell adhesive properties and associated with differences in *in vivo* tumorigenicity were also associated with functional differences in cell growth, death and survival behavior *in vitro* between the two cell lines.

Identifying the differences in adhesion-regulated signaling pathways that determine the transitions from a normal to metastatic phenotype may have important applications in the understanding of SCLC pathogenesis and thus possible treatment strategies.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell lines and culture conditions

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Md. USA). The human SCLC cell line (NCI-H69) (198), and the feline thymoma cell line 3201 were maintained in RPMI 1640 whereas the squamous carcinoma cell lines (A431, SCC-15) and mouse kidney tubule (MKT) cells were maintained in MEM (Gibco/Invitrogen, Mississauga, ON, Canada). Rat-2 untransformed and Rat-2-V-H-Ras transformed fibroblasts were kindly provided by Dr. James Stone and maintained in RPMI. Madin-Darby canine kidney (MDCK) cells were maintained in DMEM (199). All media were supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin/ streptomycin (Gibco/Invitrogen) at 5% (v/v) CO_2 at 37^0C. For growth of H69C and H69B cells on ECM substrates, exponentially growing cells were harvested and plated on Biocoat matrix proteins 6-well plates (Becton-Dickinson, Bedford, MA) at a density of 5 x 10^4 cells /well in serum-free RPMI media. Cell morphology and survival were monitored by observation under an inverted microscope, over several days.

2.2 Induction of differentiation of NCI-H69 SCLC cells

Cultures of NCI-H69 were treated with 12 μM 5-bromodeoxyuridine (BrdU) (Sigma, St. Louis, Mo., USA). BrdU treatment led to the appearance of substrate-adherent cells within a few weeks. At that point, the non-adherent cells were removed and the adherent cells termed H69B, were grown, subcloned by limiting dilution and further propagated in the absence of BrdU.

2.3 Electron microscopy

For electron microscopy, non-adherent cells were pelleted and adherent cells were grown on coverslips. Cell pellets, or coverslips were rinsed with PBS, fixed in a freshly made solution containing 1.25% (v/v) glutaraldehyde, 1% (v/v) OsO_4 in 50 mM PIPES, pH 7.2 for 5 min at room temperature, processed for electron microscopy as described (199) and examined using a Phillips 410 transmission electron microscope.

2.4 DNA fingerprinting

H69C and H69B cultures were maintained in 75 cm^2 tissue culture flasks. Adherent cells were trypsinized, and both cell lines pelleted by centrifugation. Genomic DNA was isolated with DNAzol (Gibco/Invitrogen) according to the manufacturer's protocol. Random Amplified Polymorphic DNA (RAPD) oligonucleotide primers (random 10-mers) were synthesized by the Nucleic Acid-Protein Service Unit (University of British Columbia, CA). Reactions were carried out using the GeneAmp Kit (Perkin-Elmer, NJ, USA) and 2 ng of genomic DNA according to the manufacturer's protocol. Equal amounts of total genomic DNA from control cell lines A431, SCC-15, 3201 and MKT were included in each experiment. PCR products were resolved by electrophoresis on a 1.4% (w/v) agarose gel and visualized by ethidium bromide staining. Photographic images of the gels were captured on a GelDoc System (BioRad).

2.5 Antibodies and reagents

Table 1 lists the antibodies and fluorescent probes used in this study. The appropriate dilutions for each antibody in various assays are also indicated.

Table 1. Reagents, antibodies, fluorescent probes and their respective dilutions in specific assays.

Reagents:	Assay Type: IF	WB	FACS	Source/ Reference:
Hoechst 33258	1mg/mL	-	-	Sigma, St. Louis, Mo., USA
Phalloidin-TRITC	1:750	-	-	Sigma
Primary Antibodies				
Cytokeratin:				
AE1/AE3 (CK 1-6,8,10,14,15,16,19)	1:100	-	-	Roche Biochemicals, USA
pan cytokeratin (CK1,4-6,8,10,13,18-19)	1:100			Sigma
IH5 (mouse ascites) (cytokeratin type II)	undiluted			♦DSH B, Iowa City, IA, USA
Vimentin (VIM)	1:100	-	-	Roche Biochemicals
**pan-cadherin (N-cadherin)	1:100	1:1000	-	Sigma
*E-cadherin (HECD)	-		-	Zymed, San Fransisco, CA,USA
E-cadherin (3G8)		undiluted		(154)
*α-catenin	1:100	1:1000	-	Sigma
*β-catenin	1:100	1:1000	-	Sigma
*plakoglobin	1:50	-	-	(154)
plakoglobin (Clone 15)	-	1:1000	-	Transduction Laboratories, BD, USA
Vinculin (MAB1674)	1:100	-	-	Chemicon , USA
N-CAM (123C3)	1:100	1:100	-	Santa Cruz Biotechnology, CA, USA
N-CAM (HNK-1)	-	-	1:100	Sigma
Integrins				
α2 integrin subunit (P1E6)	1:100	-	1:100	Life Technologies, USA
α3 integrin subunit (P1B5)	1:100	-	1:100	Life Technologies
α4 integrin subunit (P4G9)	1:100	-	1:100	Telios, USA
α5 integrin subunit (P1D6)	1:100	-	1:100	Life Technologies
α6 integrin subunit (GOH3)	1:25+	-	1:100	Chemicon, USA
αv integrin subunit (anti-human αv)	1:100	-	1:100	Telios, USA
β1 integrin subunit (4B4)	1:40+	-	1:100	Coulter Electronics, Miami, Fl, USA
β2 integrin subunit (P4H9)	1:100	-	1:100	Telios, USA
β4 integrin subunit (3E1)	1:100	-	1:100	Telios, USA
Tetraspanins				
CD9 (50H.19)	1:100	-	1:100	(152)
CD63 (IA44)	1:100	-	1:100	(152)
CD81 (JS64)	1:100	-	1:100	Coulter Electronics
Secondary Antibodies				
Isotype control IgG	1:100	-	-	
FITC-conjugated goat anti-mouse IgG	1:50+	-	-	Jackson, West Grove, PA, USA
FITC-conjugated sheep anti-mouse IgG	1:50	-	-	Roche Biochemicals
FITC-conjugated Anti-rat IgG	1:50+	-	-	Roche Biochemicals
FITC-conjugated Anti-rabbit IgG	1:50	-	-	Roche Biochemicals
Rhodamine-conjugated Anti-mouse IgG	1:50	-	-	Roche Biochemicals
*HRP-conjugated Anti-rabbit IgG	-	1:15,000	-	Amersham, Baie d'Urfe, Qc, Canada
*HRP-conjugated Anti-mouse IgG	-	1:15,000	-	Amersham

*WB: primary antibodies were diluted 1:1000 in 5% (w/v) dried skim milk/ TBST; secondary antibodies were used at a dilution of 1:15,000 in 5% (w/v) milk/ TBST.

*Although this pan-cadherin antibody was generated using a highly conserved region in the intracellular C-terminal end of the classical cadherins, it has previously been shown to specifically recognize N-cadherin (155).

+ for IF all dilutions were made in PBS + 0.2% (w/v) BSA.

♦ DSHB; Developmental Studies Hybridoma Bank

2.6 Flow cytometry analysis

To obtain cells in an exponential phase of growth, cultures of suspension and adherent cells were maintained at a non-limiting density 24h before each experiment. Adherent cells were gently dissociated from the flask by treatment with Ca^{2+}- and Mg^{2+}-free PBS containing 1 mM EDTA. Cells in suspension, or dissociated adherent cells, were collected by centrifugation and washed in PBS. For each analysis, $5x10^5$ cells were incubated in 50 mL of PBS/ 0.2% (w/v) BSA containing appropriate dilutions of each antibody (Table 1) on ice for 30 min. Cells were fixed in 1% (w/v) ice cold paraformaldehyde/PBS prior to fluorescence analysis on a FACScan (Becton Dickinson, CA, USA). The mean fluorescence produced by staining with an independent isotype-matched mouse monoclonal antibody was used as control for each experiment. Histograms were generated by analyzing at least 10,000 cells/ experiment. For each analysis, parallel aliquots were taken to assess cell viability by staining with Trypan blue (Sigma). Exclusion of this dye from live cells by active transport processes distinguishes them from dead cells which take up the dye.

2.7 Metabolic pulse-chase labeling, cell fractionation and immunoprecipitation

Cells passaged 48 hrs prior to each experiment, were grown to a density of 5×10^6 cells/ T25 and pre-incubated for 2hr at 37°C in methionine-cysteine-free RPMI (ICN Biochemicals). Cells were then metabolically labeled for 1 hr with 250 mCi/ mL of TRAN ^{35}S Label (1,000 Ci/ mmol) (ICN Pharmaceuticals, Inc., Irvine, CA and chased for various periods of time with complete RPMI medium containing 10,000 fold unlabeled

methionine/cysteine before lysing in an ice-cold 0.3% (w/v) CHAPS extraction buffer (137 mM NaCl, 1 mM EGTA, 50 mM Tris-Cl, pH 7.6) which included the protease inhibitors, 1 mM phenylmethylsulfonyl fluoride and 100 mg/ mL aprotinin (Sigma). The crude extracts were clarified by centrifugation and supernatants from each time point were balanced to obtain equal amounts of radioactivity. After several rounds of preclearing using mouse serum agarose (Sigma) and protein A-Sepharose CL-4B (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) to remove non-specific adsorbing materials, the supernatants were used for immunoprecipitation with specific antibodies against integrin α_2, α_3, α_5, α_6 or β_1 subunits or the tetraspanins, CD9 and CD63. An appropriate secondary bridging antibody, eg. goat anti-mouse IgG (Jackson), was added to enhance the formation of antibody-protein complexes which were then adsorbed on a 1:1 mixture of protein A/protein G-Sepharose beads. Unbound proteins were removed by washing the pellets several times with detergent wash buffer (0.5M NaCl, 5 mM EDTA and 50 mM Tris-Cl, pH 7.6) followed by one detergent-free wash and elution into non-reducing Laemmli sample buffer (200). Immunoprecipitate samples were resolved by a 9-15 % (w/v) gradient SDS-PAGE, and visualized by PPO-DMSO fluorography. Radioactive protein markers (Amersham) were used as standards for molecular weight determination. Unlabelled H69C and H69B cells were processed for immunoprecipitation as described above. Immunoprecipitates were resolved by a 9-15 % (w/v) gradient SDS-PAGE, transferred to NitroPure membranes (Osmonix/MSI, Westborough, Mass., USA) and then processed for immunoblotting.

2.8 Preparation of total cell extracts

For total cell extracts, cells were solubilized directly in 100mm tissue culture dishes using 600 µL of hot 2x SDS Sample Buffer (200). Lysates were boiled for 10 minutes and the protein concentration measured using the bicinchoninic acid (BCA) reagent (Sigma). 100 µg/ lane of total cellular proteins were resolved on SDS/ 7.5% (w/v) polyacrylamide gels, transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) and then processed for immunoblotting.

2.9 Immunoblotting

Membranes were blocked with 5% (w/v) dried skim milk in TBST (10 mM Tris-HCl, 10 mM NaCl, 1% (v/v) Tween 20) for 1 h at room temperature and incubated with the appropriate antibodies overnight at 4°C followed by a 1h incubation with a species-specific secondary antibody (201,202). Integrin-tetraspanin immunoprecipitates were analyzed by immunoblotting with HRP-conjugaged anti-CD9 or anti-CD63 (kindly obtained from Dr. Shaw) (203). The membranes were developed with ECL reagent (Amersham Pharmacia Biotech).

2.10 Immunofluorescence

For indirect immunofluorescence staining, the H69B adherent cells were grown on glass coverslips. H69C cells were grown in suspension and attached to glass slides using a cytocentrifuge prior to each assay. Coverslips were rinsed with PBS, fixed in 3.7% (v/v) paraformaldehyde for 15 minutes, permeabilized with 0.2% (v/v) Triton X-

100 for 2 minutes on ice and blocked for 30 min at room temperature in PBS containing 3% (v/v) normal goat serum and 50 mM NH_4Cl. Coverslips were then incubated for 1 hr at room temperature with various primary antibodies, followed by a 1 hr incubation at room temperature with an appropriate secondary antibody or TRITC-phalloidin, at the dilutions indicated in Table 1. Coverslips were washed and nuclei stained with Hoechst 33258. Coverslips were washed with PBS, mounted in Elvanol (204) containing 0.2% (w/v) paraphenylene diamine (PPD, pH 8.6) and viewed with a 100x objective using a BX50 Olympus immunofluorescent microscope.

2.11 Cell Adhesion Assay

Flatbottom 96-well plates (Costar, Cambridge, MA) were coated with 100 µL solutions of 10 mg/ mL Poly-L-Lysine, collagen type I, collagen type IV, or fibronectin (Sigma) in PBS and incubated overnight at 4°C. The coated plates were then rinsed and blocked with 100 µL of 1% (w/v) BSA/ RPMI 1640 for 1 hr at 37°C followed by a final rinse in wash buffer (0.03% (w/v) BSA in RPMI). For the assay, adherent cells were harvested with 0.5 mM EDTA in Puck's saline (5 mM KCl, 137 mM NaCl, 4 mM NaHCO3), washed and resuspended at 6×10^6 cells/ 50 mg calcein acetoxymethyl (AM) ester (Molecular Probes, Eugene, OR) in 1.2 mL RPMI/0.03% (w/v) BSA. After labeling for 30 min at 37°C, cells were washed two times, and resuspended at 5×10^5 cells/mL in RPMI/0.03% (w/v) BSA. As calcein AM is non-fluorescent until hydrolyzed by intracellular esterases, total fluorescence is proportional to the number of cells in each well. The cells were plated on the substrate at 5×10^4 cells/well, incubated for 1 hr at 37°C, and then washed twice with wash buffer. Unwashed control wells were used to

determine the fluorescence of the total cells added. Percent binding was calculated as a ratio of fluorescence in the washed wells out of the total fluorescence in unwashed wells. Fluorescence was determined using a Millipore Cytofluor 2350 plate reader.

2.12 Anoikis assay and analysis of DNA degradation

Tissue culture dishes (Falcon, Becton Dickinson) were coated with a film of polyhydroxyethylmethacrylate (polyHEMA) (Sigma). Briefly, a solution of 120 mg/mL of polyHEMA in 95% (v/v) ethanol was mixed overnight, centrifuged at 2,500 rpm to remove undissolved particles, and diluted 1:10 with 95% ethanol. 0.95 mL/mm^2 were pipetted in dishes of 100 mm diameter, and dishes were left to dry at room temperature. Prior to their use, polyHEMA-coated dishes were rinsed twice with PBS.

Cell apoptosis in normal or serum-depleted cultures in the presence or absence of polyHEMA was determined using a DNA fragmentation assay (205,206). Untreated cells at each time point were included as a negative control. MDCK cultures were included as a positive control for induction of apoptosis. To induce apoptosis, H69C and H69B cells were treated with 1 mM staurosporine (Sigma) for various time points. To compare the degree of resistance to apoptotic stimuli between the two cell lines, cells were examined for DNA laddering at numerous time points after staurosporine treatment, using the fragmentation assay, as a hallmark of apoptosis. Staurosporine 1 mg/mL was reconstituted in tissue culture sterile DMSO (Sigma) and diluted to 1 mM with serum-free RPMI. Exponentially growing cells were harvested, counted for cell viability, and plated at a concentration of 1 x 10^6 cells/well into 35 or 100 mm^2 tissue culture dishes (Falcon) for the hours indicated in each experiment. Adherent cells were detached by a brief incubation in Trypsin/EDTA (Gibco/Invitrogen). At each time point, culture

supernatants (media + PBS rinse) containing any dead or detached cells were collected and centrifuged to pellet cells. Cell pellets were resuspended in PBS, transferred to a microcentrifuge tube and repelleted. For the DNA fragmentation assay, cell pellets were sheared with a Hamilton syringe needle in 35 μL of Proteinase K lysis buffer (0.5 mg/mL Proteinase K (Sigma), 50mM Tris-Cl ph 8.0, 0.5% (w/v) sodium lauryl sarkosinate (N-Laurylsarcosine sodium salt L-5125 Sigma), 10mM EDTA). Samples were incubated for 1 hour at 50°C followed by addition of 5 μL of 10 mg/mL RNAse A solution (DNAse-free, Roche Biochemicals) for 1 hour at 50°C. Orange G DNA loading buffer (1.3% (w/v) Ficoll, 0.02% (w/v) Orange G, 0.35 mM EDTA) was added and samples were electrophoresed on 1% (w/v) agarose gels.

Apoptosis was also assessed by staining with Hoechst 33258 (Sigma). Control and staurosporine-treated H69B cells grown on coverslips or cytospin preparations of H69C cells were fixed with 3.7% (w/v) paraformaldehyde fixation for 15 minutes followed by permeabilization for 2 min with 0.1% (v/v) Triton X-100. The preparations were stained with Hoechst 33258 (1 mg/ mL) for 15 min, washed, mounted and examined by immunofluorescence microscopy.

2.13 Microculture Tetrazolium (MTT) Assay

MTT (Sigma) is a water-soluble tetrazolium salt that, when taken up by metabolically viable cells, is reduced to a coloured, water-insoluble formazan salt by functional dehydrogenases. As a consequence, it is an indirect measure of cell viability (207). Exponentially growing control or serum-depleted cultures in the presence or absence of polyHEMA were harvested and plated at a concentration of 5×10^3 cells/well

onto 96-well flat-bottom microtiter plates (Costar). The MTT stock solution was prepared at a concentration of 5 mg/mL in Dulbecco's PBS and stored at 4°C. Cells prepared as above were cultured for 1-7 days at 37°C. At each time point, 50 μL of 1 mg/mL MTT solution in serum-free RPMI, was added to each well. After a 5-hr incubation, the supernatant was removed from the wells, 150 μL of DMSO (Sigma) was added to dissolve the MTT-formazan product, and the absorbance at 540 nm was measured with a microplate reader.

2.14 Soft Agarose In Vitro Tumorigenicity Assay

The double layer soft agarose growth assay was used. Cultures were split or passaged 1:2 one day prior to the experiment to obtain exponentially growing cultures of H69C, H69B, or Rat2 transformed (positive) and untransformed (negative) fibroblast control cells. Cells in suspension, or adherent cells, harvested with Trypsin/EDTA to obtain single-cell suspensions, were washed once in serum-free RPMI and counted using Trypan Blue dye exclusion. 10^5 viable single cells were added to pre-warmed 0.3% (w/v) agarose in complete RPMI and 3 mL cell suspension pipetted on a pre-formed 0.6% (w/v) agarose underlay in 35 mm plates (Falcon) and incubated at 37°C. One mL of complete growth medium was added to each dish on the next day. Colony formation was examined at least twice a week using an inverted phase contrast microscope. Colonies consisting of more than 20 cells could be identified after 1week for the positive Rat2-V-H-ras transformed fibroblast control cells. Numbers of colonies were counted in triplicate under an inverted microscope at a magnification of 40x. Aggregates of more than 40 cells were considered to be colonies.

2.15 Plasmid preparation

Constructs encoding the full-length human CD9 (pcDNA-CD9) and CD63 (pcDNA-CD63) were kindly provided by Dr. Andrew Shaw (Cross Cancer Institute) and Dr. Walter Dixon (University of Alberta) respectively. Mutagenesis of the wildtype-CD63 constructs were carried out by Renate Meuser using a site-directed mutagenesis kit, Altered Sites (Stratagene), to generate mutations in the conserved Glycine-Tyrosine dipeptide motif present in the cytoplasmic tail (section 6.1.2 and Appendix IIA). This motif is present in lysosomal membrane glycoproteins and is thought to be both an intracellular sorting signal and an internalization signal in endocytotic pathways (208). The tyrosine residue of this motif was mutated to either a glycine or a phenylalanine residue. CD9/ CD63 in Bluescript (Promega) were excised and linearized with Pvu I and gel purified before ligating into pcDNA vector (Invitrogen) in preparation for cell transfection.

2.16 Transfection of H69C cells

H69C cells were harvested at 50% confluency, washed three times and resuspended at 1 x 10^7 cells/mL in cold serum-free RPMI. 0.8 mL of cells were electroporated at 5 x 10^6 cells/mL, 240 V, 960 mF in serum-free medium with 100 μg of pcDNA (control vector) or pcDNA-CD9 or the various human pcDNA -CD63 constructs. After 3 days, H69C cells were exposed to 0.4 mg/mL G418 (Gibco/Invitrogen). G418-resistent cells were subsequently maintained in G418 containing medium until cells were analyzed for expression of mRNA using Northern blotting and RT-PCR techniques.

2.17 RNA extraction and Northern blot analysis

Total RNA was extracted from cultured cells using Trizol reagent (Gibco/Invitrogen). RNA (15 µg / cell line) was fractionated on a denaturing 1% (w/v) agarose/0.66M formaldehyde gel and transferred onto a Nitropure nitrocellulose membrane (Osmonix/MSI laboratories) using capillary diffusion. RNA was fixed to the membrane by baking in a vacuum oven for 2 hours at 80°C. Membranes were pre-hybridized for 2 hours at 65 °C in hybridization buffer containing 6x SSPE (0.18M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 0.5% (w/v) sodium dodecyl sulfate (SDS), 5x Denhardts solution (0.5 g Ficoll 400, 0.5 g polyvinylpyrrolidone (PVP), 0.5 g bovine serum albumin). cDNA probes for CD63 were prepared by excision of the pcDNA 3.1-CD63 construct by digestion with EcoRI and BamHI and gel purification of the CD63 fragment. CD63 cDNA probes were ^{32}P-random primer labeled by nick translation (Random Primers DNA Labeling System, Invitrogen) using 50 µCi of [α ^{32}P]-dATP (800 Ci/mmol, Amersham). After adding 1 mg of transfer RNA, the labeled probe was boiled for 2 minutes, cooled on ice and added to fresh hybridization buffer. The membranes were incubated in hybridization buffer overnight (16-20 hours) at 65°C. Following hybridization, membranes were washed 3 times with 2x SSPE and 0.1% (w/v) SDS wash solution for 10 minutes at room temperature followed by 1 wash for 20 minutes at 65 °C with 0.1x SSC and 0.1% (w/v) SDS. Membranes were exposed to Kodak X-Omat AR film (Eastman KODAK, Rochester, NY) using an intensifying screen at -80°C, and developed using the Kodak M35A X-OMAT processor.

2.18 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

1 μg of total RNA from H69C-CD63 transfectants was reverse transcribed using Expand™ RT (Roche) and 10 μM of an oligo dT15 primer (Applied Biosystems). RNA was denatured by incubation for 10 minutes at 65°C. Following the addition of 1 mM each of dNTP's (Roche), 5x RT buffer (100 mM Tris-HCl pH 8.3, 150 mM KCl, 6 mM $MgCl_2$), 10 mM DTT and 50 U of Expand RT, the mixture was incubated at 42°C for 1 hour. RT-PCR reactions were performed in a Perkin Elmer DNA Thermal Cycler using 0.5 mL Gene-AmpTM PCR reaction tubes (Perkin Elmer). 20 μL of RT template was diluted to 60 μL and then 1, 2.5, 5 and 7.5 μL were diluted to 10 μL and used in the PCR reaction. PCR primer pairs were designed from the coding sequence of CD63: 5' CAGCCATGGCGGTGGAAGGA 3' and 3' GGAGTCGGAGGAGTAGACCC 5'. PCR reactions were carried out in a reaction volume of 50 μL containing: 2 U of Taq polymerase (Gibco/Invitrogen), 0.5 μM each of CD63 sense and antisense primer, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.25 mM $MgCl_2$ and 0.2 mM dNTP. After an initial denaturation step at 94°C for 4 minutes, the cDNA was amplified for 35 cycles (denaturation at 94°C for 1 minute, annealing at 43°C for 1 minute and extension at 72°C for 2 minutes).

CHAPTER 3 TRANSDIFFERENTIATION IS CONCURRENT WITH CHANGES IN CYTOSKELETON AND CADHERIN-MEDIATED CELL-CELL ADHESION COMPLEXES[1]

3.1 Introduction

As discussed in the Introduction, the importance of cell-cell adhesion in differentiation and in the maintenance of the differentiated phenotype is well established. (41). In this capacity, cadherins are essential for the maintenance of integrity and function of epithelia (27). Classical cadherins are linked to the actin cytoskeleton in adherens junctions via the cytoplasmic plaque proteins α-, β-, and γ-catenin (plakoglobin) (33,209,210), vinculin and α-actinin (211). In addition to their structural role, these adhesion complexes are involved in the transduction of signals across membranes (25,26,212). Modulation of the components in junctional complexes and their signaling pathways determines cell shape, regulates cell proliferation and motogenic behavior (34). Cell adhesion molecules of the immunoglobulin superfamily are also important morphoregulators involved in cell-cell communication and in determining tissue specificity (Fig. 4) (27). N-CAM is a highly and variably sialylated transmembrane glycoprotein which plays a role in embyronic development and is present in adult neuronal tissues and in certain tumors (213). In this regard some tumors, including

[1] A version of this chapter has been published: Gilchrist, A. J., Meuser, R., Turchinsky, J., Shaw, A. R., Pasdar, M., and Dixon, W. T. (2002). Cell adhesion-mediated transformation of a human SCLC cell line is associated with the development of a normal phenotype. *Exp Cell Res* 276:63-78.

SCLC, express a fetal-like sialyated form of N-CAM (214). Due to their differential expression during morphogenesis and role in invasion and metastasis, several studies have examined the expression of cell-cell adhesion molecules in SCLC (213). Although N-cadherin and catenins have been detected in SCLC cell lines (213,214), N-CAM is the major cell-cell adhesion molecule expressed in nearly all SCLC lines (213,214). The ability of SCLC to undergo heterogeneous differentiation is a major contributing problem in the development of treatment-resistant metastatic cell populations (4). Understanding the role of adhesive complexes in regulating and defining SCLC behavior may aid in the development of successful strategies for therapeutic intervention.

In this chapter, changes in cell-cell adhesive complexes in SCLC were examined with respect to the changes in morphology and development of a differentiated phenotype. An experimental system was developed in which the highly metastatic SCLC cell line H69C was transdifferentiated towards an epithelial phenotype by treatment with BrdU. The parental H69C and derived H69B cell lines were compared using DNA fingerprinting (RAPD) analysis to rule out the possibility of cell cross-contamination. The phenotypic changes from non-adherent to adherent were characterized with respect to the level and localization of various junctional proteins and formation of intercellular junctions.

3.2 Results

3.2.1 Morphological characterization of the parental H69C SCLC cell line and the adherent H69B subpopulation.

BrdU has been used to induce differentiation in a variety of neuroendocrine tumor cell lines including melanoma, neuroblastoma, and small cell lung carcinoma (215,216). When cultured *in vitro*, classic SCLC cells grow as floating cell aggregates (Fig. 8A). BrdU treatment of the classic SCLC cell line NCI-H69, hereafter referred to as H69C, induced the appearance of an adherent epithelioid subpopulation, termed H69B for 'BrdU-treated' (Fig. 8B). The striking morphological difference between the cluster-forming H69C cells and the substrate-adherent monolayer of H69B cells was further examined using electron microscopy. Electron micrographs of H69C cells showed nuclei with scant cytoplasm and an abundance of secretory granules of varying sizes, consistent with the previously reported (7) neuroendocrine secretory nature of SCLC cells (Fig. 8C). In contrast, H69B cells had high cytoplasmic to nuclear ratios (Fig.8D). While examination of the H69C cell aggregates showed close apposition of the intercellular membranes (Fig. 8C), there was no ultrastructurally recognizable junctions. In contrast, electron micrographs of the confluent adherent H69B cells showed the presence of typical cell-cell adhering junctions, consistent with an induced epithelioid phenotype (Fig. 8D). Thus, treatment of the H69C cells with BrdU, resulted in the development of a morphologically distinct subpopulation of adherent cells.

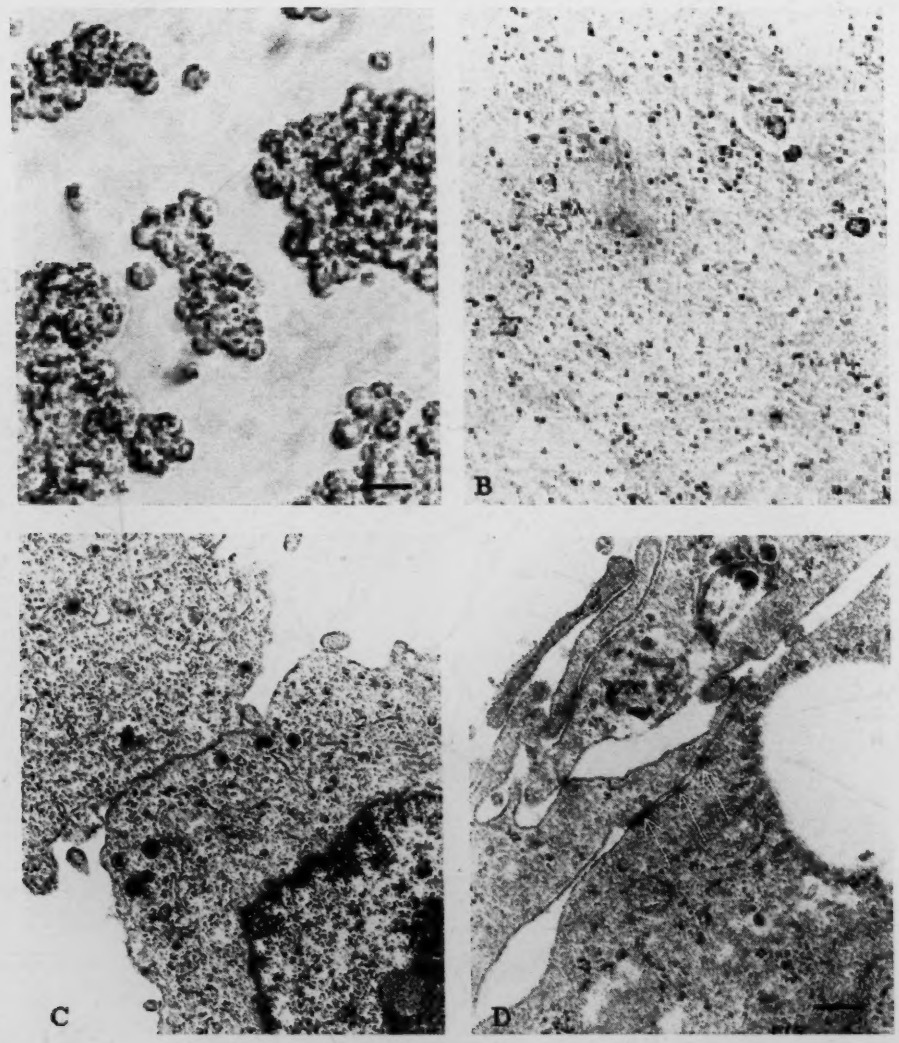


Figure 8. Morphology and ultrastructural characterization of SCLC H69C and BrdU-derived H69B cells. H69C cells were treated wtih BrdU as described in Materials and Methods to develop the stable adherent H69B cell line. Phase contrast micrograph of H69C cell aggregates **(A)** and H69B cell monolayer **(B)**. Magnification, 250x. Electron micrographs of H69C cells **(C)** and H69B cells **(D)**. Arrows in D point to the adhering junction. Bar: 1 μm.

3.2.2 H69C and H69B SCLC cells show genetically identical DNA fingerprint profiles.

In order to ensure that the H69B subpopulation resulted from induced differentiation and not contamination by a different cell type in the original parental cells, genomic DNA was isolated from both cell lines and analyzed using the RAPD technique (217,218). The PCR amplification profile of these two cell lines were compared with two other human epithelial tumor cell lines (A431 and SCC-15) as well as cell lines representing other species (3201 and MKT) as indicated (Fig. 9). The control cell lines provided unique banding patterns with all primer pairs tested. These patterns were distinct from those observed with H69C and H69B cell lines. However, for all primer combinations tested, the DNA banding patterns between H69C and H69B cell lines were identical. The DNA fingerprinting analysis clearly indicates that the differences in morphology between H69C and H69B cells are not due to underlying genetic differences.

3.2.3 Loss of tumorigenicity is associated with the transition in morphological phenotype

To address whether the observed phenotypic transition was associated with changes in tumorigenicity, the H69C and H69B cells were subcutaneously injected into severe-combined immunodeficient (SCID) mice (219). All of twelve mice injected with H69C cells rapidly formed large palpable tumors in less than 6 weeks (Fig. 10, right). In contrast, no tumors were observed in mice injected with H69B cells up to 6 months after inoculation (Fig. 10, left). Furthermore, histological

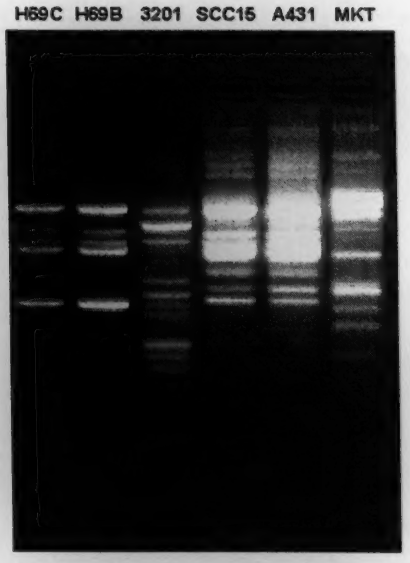


Figure 9. DNA fingerprinting of H69C and H69B cell lines. Genomic DNA was extracted as described in Materials and Methods and 2 ng was amplified with seven different random 10- base oligonucleotide primers by PCR. PCR products were separated on agarose gels and stained with ethidium bromide. The feline thymoma cell line 3201, the human squamous carcinoma cell lines (SCC15, A431) and the mouse MKT cells were included as controls for each set of primers. The data presented is representative of one set of the primers used.

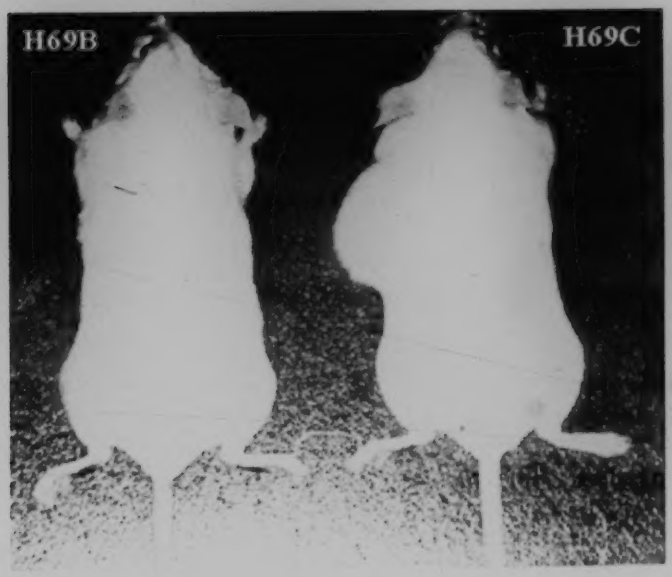


Figure 10. In vivo tumorigenicity assay of H69C and H69B cells. SCID mice were injected subcutaneously in the flanks with 1 x 10^6 H69C (right) or H69B cells (left). Mice injected with H69C cells consistently gave rise to large subcutaneous tumors (12/12) whereas mice injected with H69B cells never produced detectable tumors (0/12).

examination of tissues derived from mice injected with H69B cells revealed no evidence of any microscopic tumor growth. These results suggest that this system provides a good model to study the significance of differences in adhesive and invasive signaling pathways in SCLC cells, which define whether the cells exhibit a metastatic phenotype or not. In order to ensure that further study of the differentiated cell population was clonal and not heterogenous, single cells were isolated and subcloned by limiting dilution as described in Materials and Methods.

3.2.4 Cytoskeletal changes associated with phenotypic conversion.

To determine if the phenotypic changes observed in H69B cells were associated with differences in the level and/or reorganization of mesenchymal/epithelial marker proteins, immunofluorescent staining was carried out using antibodies against the intermediate filaments vimentin and cytokeratins (CK) as respective markers of mesenchymal and epithelial phenotypes. Cultures were established as described in Materials and Methods and processed for immunofluorescence either with a monoclonal antibody for vimentin, or a cocktail of CK antibodies, each of which recognizes a panel of cytokeratin subtypes, as listed in Table 1. Fig.11 shows that H69C cells were vimentin-positive (Fig. 11A) whereas H69B cells were vimentin-negative, with faint staining in less than 5% of cells (Fig. 11B). Cytokeratin staining appeared disorganized and primarily concentrated at the periphery thus delineating individual cells within the H69C cell aggregates (Fig. 11C). In contrast, a significant reorganization of cytokeratin filaments was observed in the H69B cells where strongly stained individual filaments could be detected, indicative of more highly organized cytoskeletal structure connecting

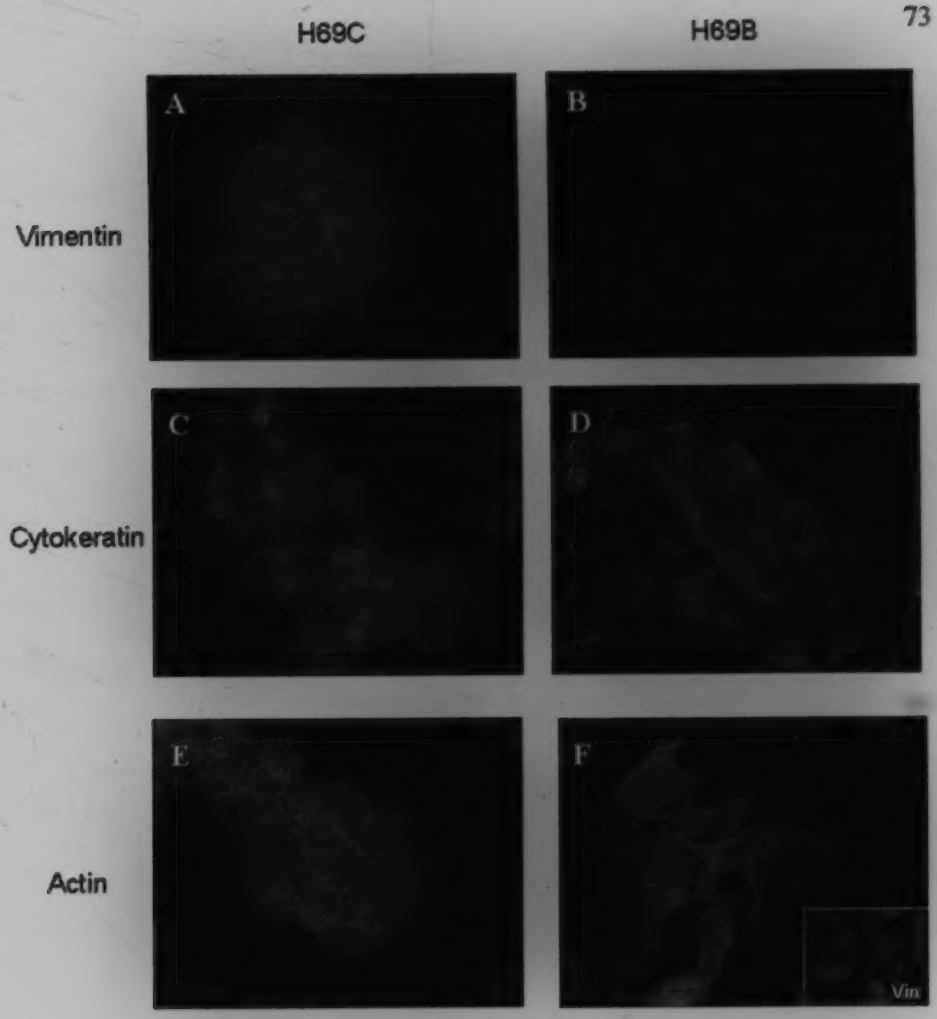


Figure 11. Organization of the cytoskeletal filaments in H69C and H69B cells. Cells were grown either in suspension or as monolayers and processed for immunofluorescence as described in the Materials and Methods. Cells were stained with anti-vimentin (A,B) or a cocktail of anti-cytokeratin antibodies (C,D) or TRITC-phalloidin in order to reveal the organization of actin microfilaments (E,F). Staining with anti-vinculin is shown in inset F. The primary antibodies were visualized with Rhodamine-conjugated anti-mouse IgG.

adjacent cells within a monolayer (Fig. 11D). Changes in the level and distribution of intermediate filaments were associated with reorganization of the actin microfilaments. The distribution of the actin in H69C cells was primarily cortical (Fig. 11E) whereas H69B cells displayed prominent stress fibers in addition to the cortical actin associated with the plasma membrane (Fig. 11F). The presence of stress fibers in these cells was further confirmed by positive staining for vinculin (Fig. 11F, inset), indicative of the formation of focal adhesion contacts. Together, the change in the staining pattern of these cytoskeletal proteins suggest a transition to a more epithelioid phenotype in the H69B cells.

3.2.5 Alterations in the protein level and organization of cell-cell adhesion molecules.

The differences in the organization of actin and intermediate filaments led to examination of the levels and distribution of the adhesion complexes in H69C and H69B cells. To this end, the presence and/or levels of N-CAM, N-cad, E-cad, α– and β–cat, and plakoglobin (pg) were determined by immunoblotting of total cell extracts (TCE) prepared from each cell line. 100 μg of TCE from H69C, H69B, and control cell lines (MDCK and A431) were processed for immunoblotting. Each experiment was repeated at least three times and the same membrane was sequentially probed with the various antibodies.

Immunoblotting with an anti-N-CAM antibody detected a major band at ~135kDa and a minor band at ~190 kDa in the H69C cells which were not present in either the H69B cells or the control MDCK cells (Fig. 12; N-CAM). Anti-N-cad antibodies detected a strong band corresponding to N-cad at ~135 kDa in the H69B cells which was

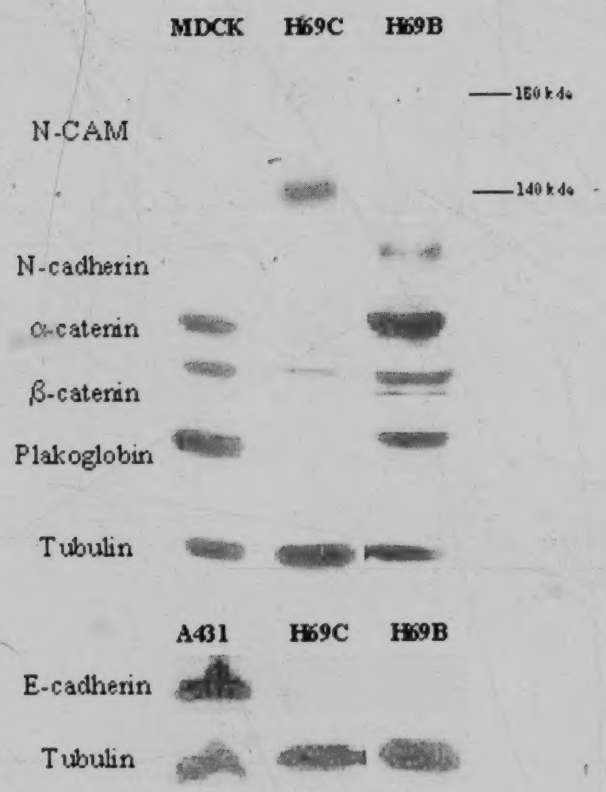


Figure 12. Levels of various cell adhesion proteins in H69C and H69B cells. Total cell lysates prepared from H69C, H69B and control cell lines (MDCK, A431) were processed for immunoblotting with antibodies specific for N-CAM, N-cadherin, E-cadherin, α-catenin, β-catenin or plakoglobin as described in the Materials and Methods. Membranes were sequentially probed with different antibodies and developed by ECL. The same membrane was probed with an anti-tubulin antibody to confirm equal loading of the total cellular protein.

undetectable in the H69C and the control MDCK cells (Fig. 12; N-cadherin). However, by increasing the amount of TCE to 300 μg for H69C, we were able to detect a distinct but faint 135 kDa band indicating that N-cad is expressed at low levels in the H69C cells and becomes significantly upregulated in the H69B cells. Processing of the membrane with an anti-E-cad antibody detected a ~120 kDa band in the control A431 cells which was not present in either H69C or H69B cells (Fig. 12; E-cadherin). A single band of ~102 kDa corresponding to α–cat was strongly expressed in the H69B and control MDCK cells whereas it was barely detectable in the H69C cell line (Fig. 12; α–catenin). Similarly, immunoblotting with the anti-β–cat antibodies detected a single band of 94 kDa in all three cell lines however, its level was significantly lower in the H69C cells (Fig. 12; β–catenin). Probing with the anti-pg antibodies revealed a barely detectable band at 82 kDa in H69C cells which was again strongly expressed in both H69B and control MDCK cells lines (Fig. 12; plakoglobin). The tubulin blots are included to verify that approximately equal amounts of total cellular proteins were loaded for each cell line.

Organization of the cell-cell adhesion proteins was examined by immunofluorescent assay. Probing with an anti-N-CAM antibody showed that this protein was strongly stained and primarily localized to the intercellular spaces of H69C cell clusters (Fig. 13A) whereas it was hardly detectable in the H69B monolayers (Fig. 13B). Staining with the anti-N-cad antibodies detected homogenous and weak distribution of N-cad in H69C cells (Fig. 13C). In contrast, N-cad staining was significantly stronger in the H69B cell line and primarily localized to the periphery (Fig. 13D). H69C cells also exhibited weak staining for α–cat (Fig. 13E), β–cat (Fig. 13G),

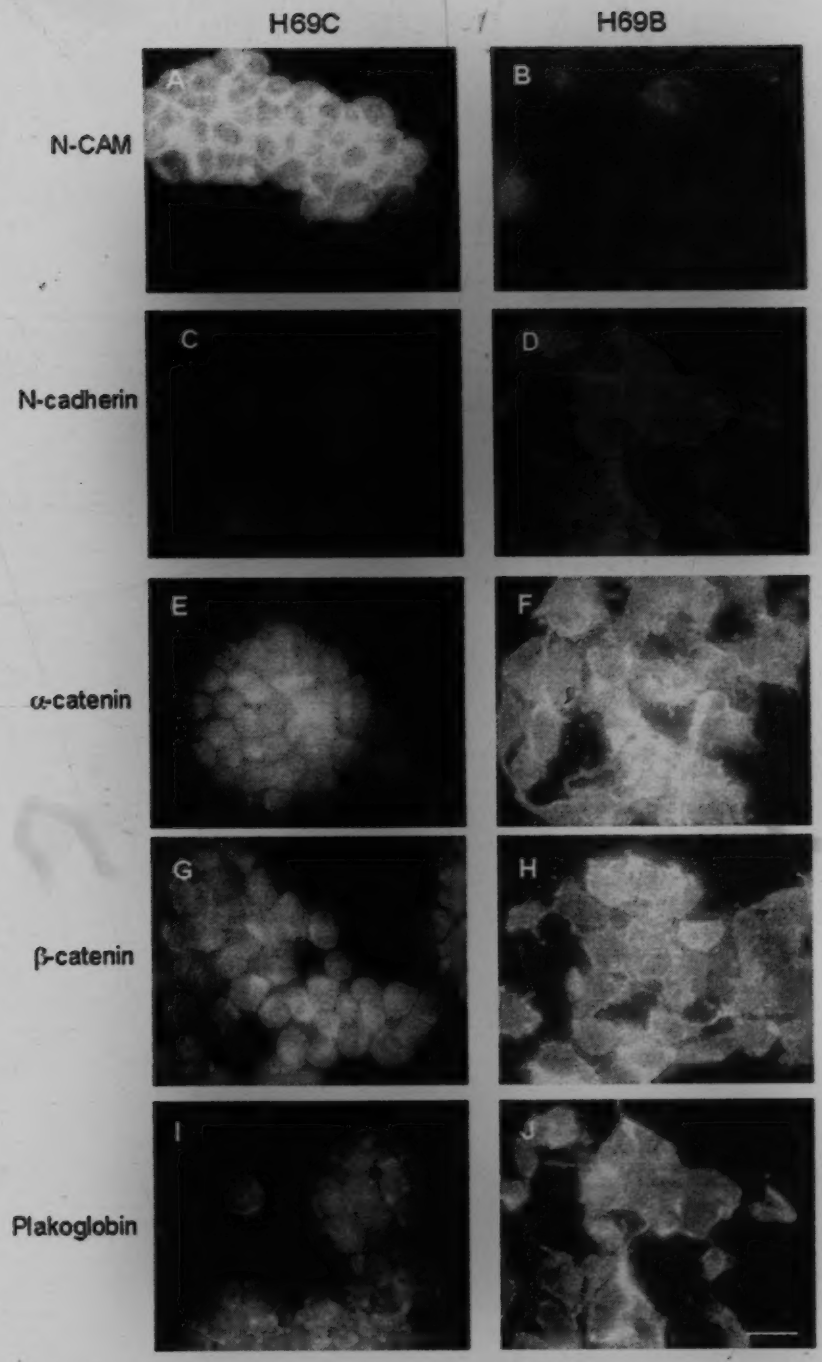


Figure 13. Subcellular organization of various cell adhesion proteins in H69C and H69B cells. Cultures of H69C and H69B cells were processed for immuno-fluorescence staining as described in Materials and Methods using antibodies to N-CAM (A, B), N-cadherin (C,D), α–catenin (E,F), β-catenin (G, H) or plakoglobin (I, J). Primary antibodies were visualized with fluorochrome-conjugated species-specific secondary antibodies. Bar, 20 mm.

and pg (Fig. 13I). The staining for these proteins was intracellular and homogenous with no detectable membrane localization. In contrast, the staining for α–cat (Fig. 13F), β–cat (Fig. 13H) and Pg (Fig. 13J) was significantly stronger and distributed both intracellularly and at the plasma membrane in H69B cells. Together, these results show that the transition to a more epithelioid phenotype was associated with increasing levels of cell-cell adhesion proteins as well as their subcellular reorganization and peripheral localization.

3.3 Discussion

3.3.1 Epithelial Plasticity of SCLC Cells

SCLC originates from multipotent pulmonary neuroendocrine progenitor cells that retain the ability to transdifferentiate into various epithelial cell lineages in response to the requirements of the microenvironment of the lung (4,7-9,11,13,17,18). This epithelial plasticity is maintained by precise regulation of epithelial-mesenchymal transitions and is required for morphogenetic remodeling of lung tissue during normal embryonic development (13). In the adult lung, EMTs occur during transient phenotypic transformations such as those required to regenerate tissues during wound healing, inflammation and injury repair (17,18). Thus lung epithelial cells must be able to dynamically downregulate the stable intercellular adhesion associated with epithelial differentiation and acquire the mesenchymal characteristics required for proliferation and invasion of ECM/connective tissues (20-22). Epithelial or mesenchymal differentiation is regulated by cell surface adhesion receptors that transduce extracellular signals to the nucleus and modulate the transcription of epithelial- or mesenchymal-associated genes (220-222). Thus, disruptions in cell-cell or cell-matrix interactions are key events which can lead to changes in cell differentiation and proliferative or apoptotic potential. Aberrant reactivation of EMTs has been observed during the progression of carcinomas and is proposed to be a mechanism for determining tumor cell invasiveness and metastatic behavior (23). In order to investigate the process of transdifferentiation and mechanisms underlying SCLC pathogenesis, we have experimentally induced a phenotypic transition towards an epithelioid phenotype in a SCLC cell line using BrdU.

This morphological transformation led to the development of an adherent monolayer (H69B) from spherical tightly aggregated cell-cell clusters of the non-substrate adherent parental line (H69C). Ultrastructural examination of the two cell types by electron microscopy, revealed the formation of ultrastructurally recognizable adhesion complexes in the adherent cells only. Using RAPD analysis we confirmed that the H69C and H69B cell types are genetically identical excluding the possibility of clonal diversity in the original line. The phenotypic stability observed following removal of BrdU suggests that the differentiated state is a cellular response requiring a change in gene expression and the effect of BrdU is to initiate molecular events underlying the differentiation response (223,224). The data in this present study suggests that changes in the expression of genes involved in determining an epithelial differentiation program are responsible for the differences in morphological and adhesive properties. Therefore, one objective in utilizing this experimental system was to characterize the expression of cytoskeletal and adhesion-related proteins that participate in structural and signaling complexes responsible for maintaining epithelial versus mesenchymal potential.

3.3.2 Phenotypic transition of SCLC cells is associated with rearrangement of cytoskeletal components.

EMT/MET transitions are associated with rearrangement of cytoskeletal components. In this study, the transition from an aggregated to an epithelioid phenotype was associated with decreased expression of vimentin and increased cytokeratin intermediate filaments and its reorganization. H69C cells have previously been shown to express vimentin and a limited cytokeratin profile, cytokeratins 8, 18 and 19, consistent with their proposed endodermal origin (6,225). Coexpression of vimentin and cytokeratin

filaments, observed in many cancers, has been correlated with increased invasive and metastatic behavior (6). In contrast, H69B cells lost vimentin expression and upregulated cytokeratins that exhibited a highly organized distribution. Thus, the phenotypic transition of H69C to H69B is accompanied by a reduction in vimentin levels and increased cytokeratins. Furthermore, H69B cells exhibited a major reorganization of the actin cytoskeleton and appearance of stress fibers, consistent with cell spreading, adhesion to substrate and development of epithelioid phenotype.

3.3.3 The SCLC cell phenotypic transition is associated with alterations in cell-cell junctions and adhesion molecules.

EMT/MET transitions are associated with coordinated changes in cell-cell junctions and adhesion molecules (21-23). The N-CAM family of closely related glycoproteins are involved in homotypic and heterotypic cell-cell interactions that is important for tissue segregation during normal development (213,214). In adult tissue, N-CAM is found on cells of the nervous system and is a marker for cells of neuroendocrine origin. Due to the large polysialyate groups on embryonic N-CAM, tight cell-cell adhesion mediated by cadherins is prevented. This is consistent with a mesenchymal phenotype which facilitates morphogenetic rearrangement of embryonic cells during development (213). N-CAM is present on all SCLC lines examined and may contribute to the invasive behavior of these cells (213,214). High levels of N-CAM in H69C cells were detected by immunoblotting (Fig. 12), immunofluorescence (Fig. 13) and flow cytometry. In contrast, N-CAM expression was undetectable in H69B cells. Thus epithelial differentiation of the H69B cells is accompanied by a reduction in N-CAM in agreement with other model systems (226-228). The significance of cadherin-mediated

cell-cell adhesion and formation of cadherin/catenin adhesive complexes, particularly E-cadherin, in the maintenance of epithelial integrity and in suppression of tumor cell invasion is well characterized (26,27,33,209,212). N-cadherin is the major cadherin found in neural tissue and is critical for the migration of neural crest cells during embryonic development (27). E-,N- and P-cadherins have been detected at low levels in SCLC cell lines and appear to have a heterogenous expression pattern (6,213,229). Examination of cadherin expression showed that H69C cells express very low levels of N-cadherin, which is diffusely distributed in the cytoplasm. In contrast, N-cadherin expression was highly elevated in H69B cells and primarily localized to peripheral membranes at the areas of cell-cell contact. E-cadherin was not detected in either cell type. Consistent with upregulation of N-cadherin expression, the levels of cadherin-associated proteins, α-/β-catenins and Pg, were greatly increased in H69B cells and re-distributed to cell-cell borders. The increased levels of catenins concomitant with the formation of ultrastructurally recognizable adhering junctions in H69B cells support the development of an epithelial phenotype. The types and levels of cadherins expressed during cell differentiation or transformation are critical events in defining mesenchymal versus epithelial behavior (22,23). While expression of E-cadherin has been linked to suppression of tumor cell invasiveness, expression of inappropriate cadherins, such as N-cadherin in epithelial cells, leads to the development of an invasive phenotype (222,230). However, in this experimental system, increased expression of N-cadherin and catenins occurs in the absence of E-cadherin. This is consistent with observations seen in squamous cell carcinoma cell lines lacking E-cadherin expression (231). This, together with the development of extensive cell-cell contacts indicates that upon increased

expression of α- and β-catenins and plakoglobin, N-cadherin is functioning to stabilize the formation of junctional adhesion complexes (231).

CHAPTER 4 CHARACTERIZATION OF CELL-MATRIX ADHESIVE COMPLEXES IN H69C AND H69B CELL LINES[1]

4.1 Introduction

Integrins serve as a transmembrane link between the ECM and the cytoskeleton playing important roles in signaling pathways that regulate proliferation, migration, differentiation, or programmed cell death (130,137). In development, integrins regulate matrix and growth-factor induced EMT/MET and ECM-induced morphogenesis of lung epithelial cells (13). Altered cell-ECM interaction is also seen in tumor progression. Tumor cell-ECM interactions may be promoted by the expression, organization and function of specific integrin receptor subtypes that dictate a cell's ability to adhere, migrate and/or invade in a tissue-specific microenvironment. As described in section 1.3.5.1, integrin function is facilitated by a variety of proteins. Although much is still unknown about the pathways and associated proteins that regulate integrin function, insights into how these proteins assist in integrin assembly, processing, transport, glycosylation state, subcellular location, and assembly in multimolecular membrane complexes are now being elucidated.

[1] Parts of this chapter have been published: Gilchrist, A. J., Meuser, R., Turchinsky, J., Shaw, A. R., Pasdar, M., and Dixon, W. T. (2002). Cell adhesion-mediated transformation of a human SCLC cell line is associated with the development of a normal phenotype. *Exp Cell Res* 276:63-78.

Integrin conformation, subcellular location, and cell surface expression is integral to integrin function (232). The conformation required for ligand recognition is the same tertiary structure required for heterodimer assembly in the ER (233). Sequences required for ligand-binding are intimately associated with the selectivity (α/β combination) of heterodimer assembly. This ensures that the specificity of heterodimer expression is specifically linked to its functional ligand-binding capability and coordinated regulation by divalent cations (110). Thus, the compatibility and success of α /β heterodimer assembly is ultimately critical for the specificity of integrin receptor function, regulation and signaling (234). Heterodimeric association is mediated through the extracellular domains of the α– and β–subunits and these associations are still maintained with truncation of the cytoplasmic domain (235). Hydrophobic and ionic interactions between conserved membrane-proximal regions of the integrin subunits also play a role in determining integrin conformation and are critical for ligand-binding activity. Truncation of the N-terminal region of the α cytoplasmic domain abolishes the ability of the integrin to reach the cell surface and in some cases results in very low levels of cell surface expression (236). Therefore, both extracellular domains and conserved cytoplasmic sequences are required for subunit association, intracellular processing and trafficking of the integrin subunits, and their subsequent transport to the cell surface.

Integrin function is not only specified by its conformation state but also results from their extensive post-translational modification which occur before reaching the cell surface. Integrin receptor processing is regulated by chaperone proteins in the ER, glycosyltransferase enyzmes, and other proteins in the Golgi. Initial post-translational modifications occur during protein synthesis in the ER. Pulse-chase labeling studies are

used to assess the kinetics of precursor formation and the time course of integrin receptor maturation. The first stage of assembly occurs in the ER where the precursor forms of the α and β_1 subunits interact to form the heterodimer. Further processing of the heterodimer occurs in the Golgi (237-239). The proper folding and assembly of pre-α and β_1 in the ER may depend on transient interactions with ER transmembrane chaperone proteins such as calnexin (240). Calnexin, an ER resident protein, was found to be associated with both the α_6 and β_4 subunit precursors (241). Although association of integrin α and β subunit precursors seems to occur independently of endoproteolytic cleavage or Golgi modifications such as glycosylation(242), both processes are major determinants of integrin function (240). Certain integrin α subunits, including α_3, α_5 and α_6, undergo proteolytic cleavage resulting in a disulfide-linked heavy and light chain conformation essential for ligand-recognition and signaling function (243,244). For example, cleavage of the α_6 subunit was demonstrated to be functionally important for integrin signaling although its ligand binding capacity and cell spreading on laminin-1 was not affected (244). The inability of uncleaved α6 subunits to propagate intracellular signals elicited by PMA in the same study may be due to its aberrant structure affecting its microclustering in the plane of the membrane.

The initial steps in N-glycosylation of β_1 integrins take place in the ER where partial glycosylation of the β_1 integrins by ER glycosyltransferases leads to formation of a high mannose 110 kDa precursor (pre-β_1) forms. Immature β_1 integrin is known to accumulate in the ER forming a stable pool and is synthesized in excess over the α chain integrin in many cell types (237,240,245). Excess β_1 precursor subunit, if not transported as an αβ heterodimer to the Golgi, is degraded in the ER or a pre-Golgi compartment

(246). Experimental evidence indicate that the maturation rate of the pre-β_1 pool is dependent on the number of pre-α subunits available which suggests that further exit of integrin precursors from the ER requires αβ-complex formation (237,238,247). Thus, heterodimer formation is regulated both by the levels of α chain and their affinity for β_1 subunit. It is possible that exposure of an export signal on the α subunit may be required for packaging and export or targeting of the heterodimer from the ER thus regulating the amount and specificity of integrin receptors that are available at the cell surface (121).

$\alpha\beta_1$ integrin heterodimers are transported to the Golgi where α and β subunits undergo further processing by Golgi glycosyltransferases to generate the fully glycoslyated mature 130 kDa form of the β_1 chain (239,248). Subcellular fractionation experiments reveal that the mature, but not the precursor β_1 isoform, is localized to the Golgi. The mature heterodimer is then transported to the cell surface where additional changes in phosphorylation state, activation state and protein associations modify integrin receptor function. Cell type-specific and conformational differences in associations with distinct α subunits are likely to result in the differential processing of β chains (237). Multiple reports have suggested that N-glycosylation of β_1 integrins is essential for optimal function (249,250). In fact, the rate of integrin processing and maturation can be altered in response to stimulation by growth and differentiation factors or oncogenic transformation (238). For example, processing of β_1 integrin was accelerated by TGF- β_1 through stimulation of Ras, which affected its cell surface expression and functional binding to ECM components (237,239). Another study found that downregulation of the focal adhesion component, talin, which binds to the β_1 cytoplasmic domain, results in glycosylation defects and abnormal maturation of the $\alpha_5\beta_1$ integrin affecting its cell

surface function (121,251,252). Through activation of specific glycoslytransferase enzymes appropriate ligand and physiological composition of the ECM environment can differentially regulate function of surface receptors such as integrins.

Integrin function at the cell surface may also be modulated by the existence of natural α and β subunit splicing variants ($\alpha_{3A,B}$; $\alpha_{6A,B}$; α_{7A-C}; β_{1A-D}; β_{3A-C}; and β_{4A-E}). These occur in the cytoplasmic domain and appear to influence localization, function and/or affinity state of the integrin (253). For example, β_{1C} variants are unable to bind talin and FAK, and as expected do not localize to focal adhesions (254). Others such as the β1D subunit in tensile muscle tissues exhibit an increased affinity for talin and filamin and result in more stabilized adhesion (255). On the other hand, the β1B subunit acts as a dominant negative inhibitor of integrin localization to focal adhesions and triggering of FAK tyrosine phosphorylation, which subsequently appears to interfere with cell spreading and migration (253). Alternate integrin splice variants have cell- and tissue-type specific expression, are upregulated in development in association with differentiation to more mature phenotypes, and therefore are likely to have functional significance (256,257). Consequently, distinct structural differences displayed by the β subunit cytoplasmic domain contribute to its functional ability to localize to focal contacts, its ability to initiate tyrosine phosphorylation and to induce signals that regulate gene expression, proliferation and motility (254,258). Mechanisms of transdominant integrin regulation also exist, in which expression and activation of specific integrins can affect the localization and activity of other integrins (259,260). Thus, as a result of ligand-specific integrin activation, the selection of which proteins are recruited to distinct

integrin heterodimer complexes defines the highly dynamic nature, composition and specialized function of focal contacts (139).

Lateral associations of integrins with transmembrane regulatory proteins are also critical determinants in integrin localization, activation, and their assembly and participation in signaling complexes at the plasma membrane (170). The tetraspanin superfamily of transmembrane glycoproteins, (discussed in section 1.3.5.2), is one such class of proteins (172). Highly conserved throughout evolution, this family is ubiquitously expressed in a distinct tissue-specific manner, on all cell types (172). The wide distribution of tetraspanins, their ability to associate with one another and other transmembrane proteins, and their presence on intracellular vesicles also suggests a role in vesicle trafficking and membrane fusion events might be associated with the diversity of their cellular functions. A number of studies support this observation. The lysosome integral membrane protein CD63 was originally described as a platelet activation marker (Pltgp40 or granulophysin) present in dense granule membranes (261-264) and is a surface marker for neutrophil and basophilic granulocyte activation (265,266). CD63 expression on the cell surface with exocytosis of granule contents can possibly be explained by fusion of lysosomal or endosomal membranes with the plasma membrane (172). Other tetraspanins have been upregulated and associated with immune cell activation. CD81, CD82 and CD53 tetraspanins trigger activation signals in T and B cells (176). Cross-linking CD53, in rat macrophages, activates a cellular response that is mediated by PKC and releases nitric oxide as a consequence of induction of nitric oxide synthase (267). CD63 was shown to associate with and upregulate CD11/CD18 integrins involved in neutrophil cell-cell adhesion in association with Lyn and Hck tyrosine kinase

activities (268). CD9 appears to stabilize the transmembrane precursor form of heparin-binding EGF-like growth factor (HB-EGF), also known as the diptheria toxin receptor (DTR), on the cell surface by preventing processing and/or internalization (269). CD9 may allow for increased translocation of DTR/HB-EGF to the cell surface (270). Alternatively, CD9 may extend the half-life of these receptors at the cell surface either by inhibiting the normal proteolytic processing that the DTR/HB-EGF transmembrane precursor undergoes to become the soluble form, or by affecting the rate of DTR/HB-EGF precursor recycling (270). Additionally, CD9 and CD81 have been shown to be involved in muscle cell fusion and maintenance of myotubules (197), sperm-egg fusion in mice and rats (CD9), and anti-tetraspanin antibody-induced blocking of virus-mediated syncytium formations (CD81, CD9) (176,271). Interestingly, many tetraspanins are receptors or associated with receptors for viruses and microbial pathogens which may reflect their role in membrane fusion events possibly in the context of lipid raft associations (176,272). That is, although yet to be established, oligomerization of various tetraspanins may function to initiate and/or regulate raft clustering and lateral assembly of protein complexes in the plasma membrane thereby initiating fusion of the virus envelope with the cell membrane (272).

The involvement of tetraspanins in determining cellular activation, adhesive and migratory status, their complex role in metastasis and the specificity of their association with integrins suggest that tetraspanins may regulate integrin function (273). One way in which tetraspanins could regulate integrin function is by targeting them to different sites within the cell or specific subdomains along the plasma membrane. Tetraspanins have been found in lipid rafts and motility-related structures (274-276). Like integrins,

tetraspanins have been found to be present and involved at junctional sites of cell-cell adhesion (277,278). For example, integrin $\alpha_3\beta_1$, CD9 and transmembrane HB-EGF have been found to co-localize at cell-cell contact sites near adherens junctions (279). In monkeys, DRAP-27 (the CD9 homolog) is co-precipitated with transmembrane HB-EGF and forms a complex (280). It may be that direct interactions of juxtacrine growth factors, receptors and cell adhesion molecules influence cell proliferation during the cell-cell adhesion process (269). TM4-$\alpha_3\beta_1$ functional complexes are present at endothelial cell to cell contacts and the tetraspanin components of the complex are thought to be able to modulate cell motility through regulation of the integrin adhesiveness (281). CD9 and CD151 are both tetraspanins shown to be involved in homotypic cell-cell adhesion in hematopoetic cell lines (282,283). In addition, CD151 has been recently found to be a novel constituent of hemidesmosomes in association with the $\alpha_6\beta_4$ integrin and may regulate the spatial organization of hemidesmosomes (284). Intriguingly, initial co-distribution of CD151 with $\alpha_3\beta_1$ in focal adhesions of basal skin keratinocytes was found to be replaced by incorporation of $\alpha_6\beta_4$ integrin and subsequent induction of hemidesmosome formation. Tetraspanins have now been linked to intracellular signal transduction pathways through their associations with signaling molecules such as phosphatidylinositol 4-kinase (PI4-K), PKC, PTPases, growth factor ligands, and small GTP binding proteins (285-290). These studies support the notion that tetraspanins may regulate integrin function by recruiting signaling molecules into proximity of integrins leading to the assembly of integrin-containing signaling complexes at the plasma membrane (273).

Studies on SCLC tumors and cell lines have determined that they have a limited repertoire of integrin and tetraspanin expression (291-294). The exact role of integrins and associated tetraspanins in pathogenic EMT leading to tumorigenesis and metastastic potential in the lung has yet to be defined. To further characterize these mechanisms in SCLC in the present study, the cell surface expression and repertoire of integrins and tetraspanins were compared between the H69C and H69B cell lines. The type, localization and level of integrins and tetraspanins were analyzed using immunofluorescent microscopy and immunoblotting. Pulse-chase labeling and immunoprecipitation of H69C and H69B were used to assess processing and turnover rate of these various proteins. These analyses revealed differences in integrin-tetraspanin associations in H69C and H69B cells. ECM-specific substrates and adhesion assays were used to assess differences in ligand-specificity between the two cell lines.

4.2 Results

4.2.1 Phenotypic transition is associated with increased levels of cell surface β_1 integrins and tetraspanins.

Changes in integrin expression and function are associated with changes in epithelial-mesenchymal status and tumor invasiveness (11,13). We examined the profile of integrins and associated tetraspanins in H69C and H69B cell lines (Fig. 14 and Table 2). Flow cytometry revealed significant differences in integrin profiles between H69C and H69B cell lines. H69C cells expressed a restricted integrin repertoire with low levels of $\alpha_3\beta_1$, $\alpha_6\beta_1$, and little or no detectable $\alpha_2\beta_1$ and $\alpha_5\beta_1$. In contrast, the adherent H69B showed increased levels of cell surface $\alpha_3\beta_1$, and $\alpha_6\beta_1$ and novel expression of high levels of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ (Fig. 14 and Table 2). There was also no detectable cell surface α_4, αV, β_2 and β_4 subunits in either of the two cell lines (169).

Examination of the surface expression of the tetraspanins in H69C cells showed extremely low levels of CD9 (4% positive cells), and moderate expression of CD63 and CD81 (40-60%; Table 2). In the H69B cells, CD9, in particular, showed a striking increase in cell surface expression from 4 to 28%. Similarly, CD63 and CD81 surface expression were also elevated in these cells (Table 2).

4.2.2 Subcellular distribution of β1 integrins and tetraspanins in H69C and H69B cells.

Subcellular organization of integrins and tetraspanins was examined by immunofluorescent staining using a panel of integrin- and tetraspanin- specific antibodies. The results obtained showed clear differences in the cellular distribution of

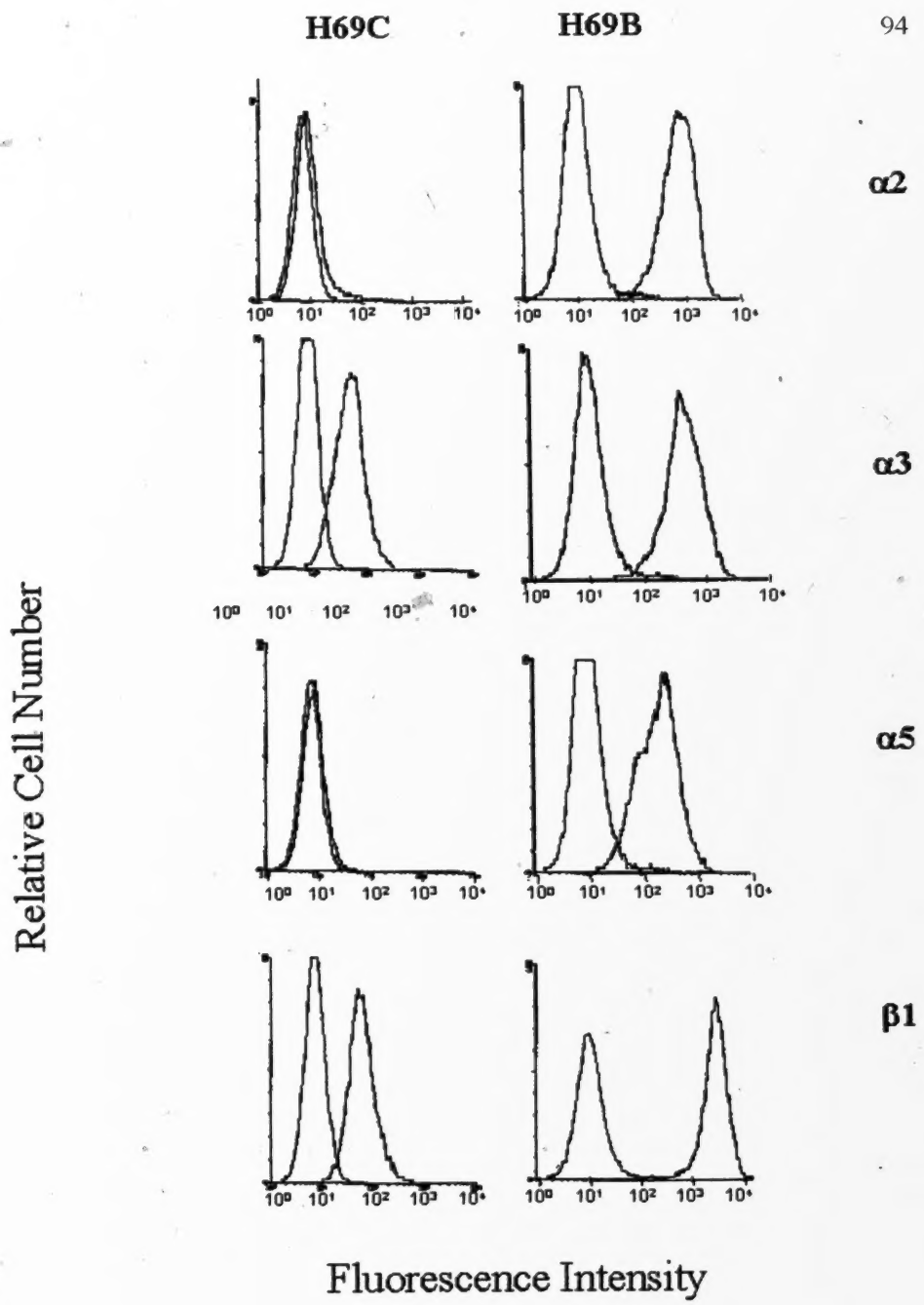


Figure 14. Changes in cell surface expression of integrins. Aggregates of H69C and monolayers of H69B cells were processed for flow cytometric analysis of α2, α3, α5, and β1 integrins as described in Materials and Methods and analyzed on a FACScan. Representative fluorescence profiles of each cell line for each antibody is presented together with the isotype-matched controls that are indicated by the left peaks in each panel. Quantitative mean fluorescence intensity (MIF) for the above analyses, as well as additional integrins and tetraspanins are presented in Table 2.

Table 2. Surface expression of integrins and tetraspanins on H69C and H69B SCLC cells.

	♣% Mean fluorescence	
	H69C	**H69B**
Integrin subunits		
α2	17.7	55.8
α3	76.1	63.2
α4	17.1	14.6
α5	21.2	86.5
α6	58.0	96.0
αv	16.9	11.6
β1	93.8	99.1
β2	17.7	8.9
β4	15.5	6.4
Tetraspanins		
CD9	4.0	28.0
CD63	40.0	86.0
CD81	60.0	89.0

♣ Cells (0.5×10^6) were incubated with primary antibodies , washed, and incubated with an anti-mouse IgG conjugated to FITC and then analyzed for fluorescence using a flow cytometer. Mean fluorescence intensity is expressed as a percentage of positive cells relative to isotype-matched IgG control.

these proteins between the two cell lines (Figs. 15 and 16). Staining of H69C cells with anti-α_2 integrin antibodies showed a weak and diffuse cytoplasmic staining (Fig. 15A). In contrast, H69B cells showed significantly stronger staining of α_2 that was found to be more concentrated in membrane processes (Fig. 15B). This reorganization in integrin distribution indicates the formation of focal adhesions in substrate-adherent cells which was verified earlier by staining for vinculin (Fig. 11F, inset). α_3 integrin in H69C cells showed moderate expression and was predominantly localized to the perinuclear region (Fig. 15C). Although there did not seem to be much difference in the total levels of α_3 between the two cell lines, the localization was significantly changed from that of a homogenously distributed pattern in H69C cells, to being primarily concentrated in the peripheral membrane processes of the H69B cells (Fig. 15D). Staining for α_5 integrin in H69C cells showed a very weak and homogenously distributed cytoplasmic pattern (Fig. 15E). In contrast, in H69B cells α_5 staining was stronger with more concentration in cell processes (Fig. 15F). Similarly, we detected a weak and homogeneously distributed staining pattern for α_6 in H69C cells (Fig. 15G) in contrast to H69B cells in which this integrin was more intensely stained (Fig. 15H). The staining for the β_1 integrin subunit in the two cell lines paralleled that observed for its partner α subunits. Here again, homogenous and cytoplasmic staining seen in H69C cells (Fig. 15I), was redistributed to a pronounced peripheral organization in the membrane processes in H69B cells (Fig. 15J).

Fig.16 depicts the subcellular distribution of CD9, CD63 and CD81 tetraspanins. In H69C, we detected weak and homogenously distributed cytoplasmic staining for CD9 (Fig. 16A) and CD81 (Fig. 16E) whereas the staining for CD63 was stronger and

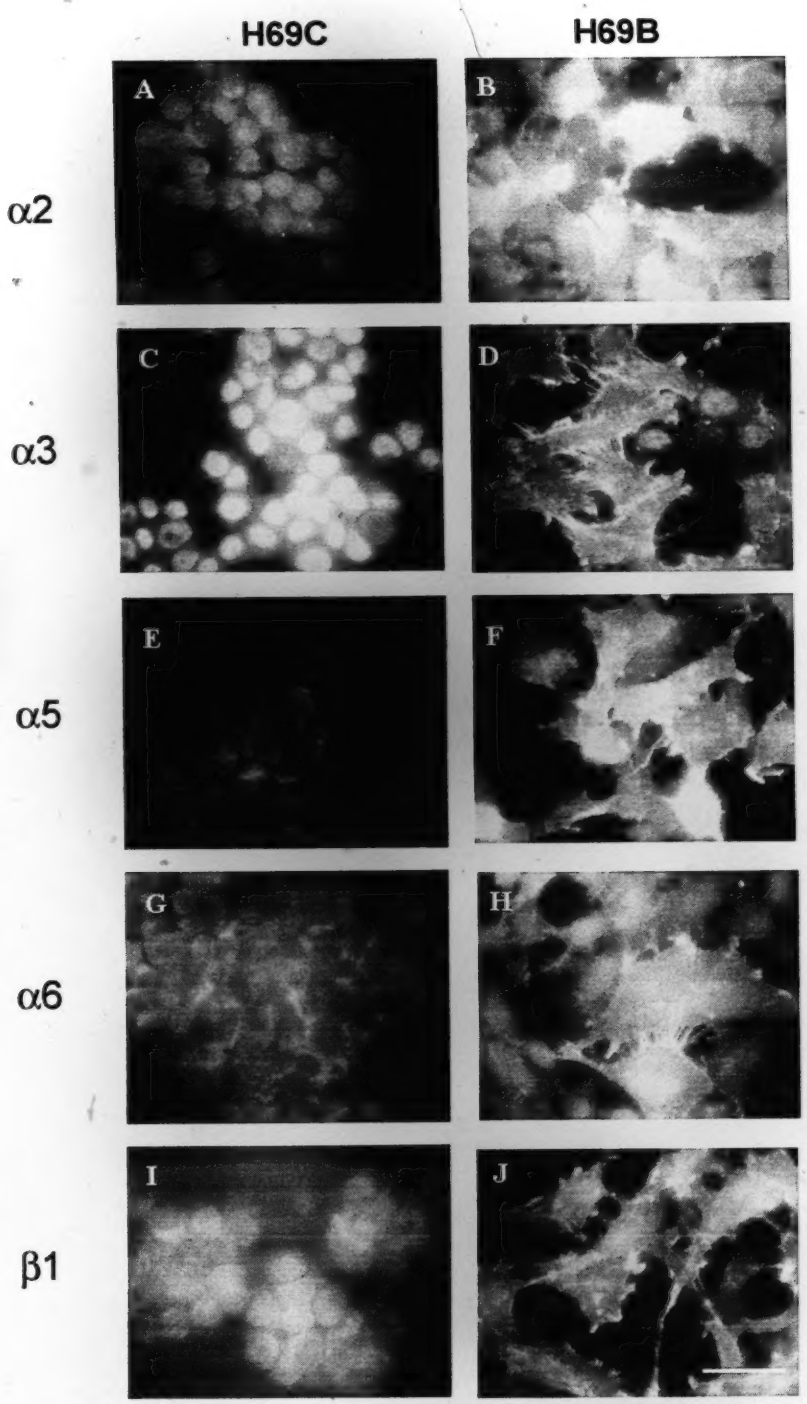


Figure 15. Changes in cellular organization of integrins following conversion to an epithelioid cell phenotype. Cells were processed for immunofluorescence staining as described in the legend of Fig. 11, using antibodies to α2 (A,B), α3 (C,D), α5 (E,F), α6 (G, H), or β1 (I, J) integrins. Primary antibodies were visualized by FITC-conjugated species-specific secondary antibodies. Bar, 20 mm.

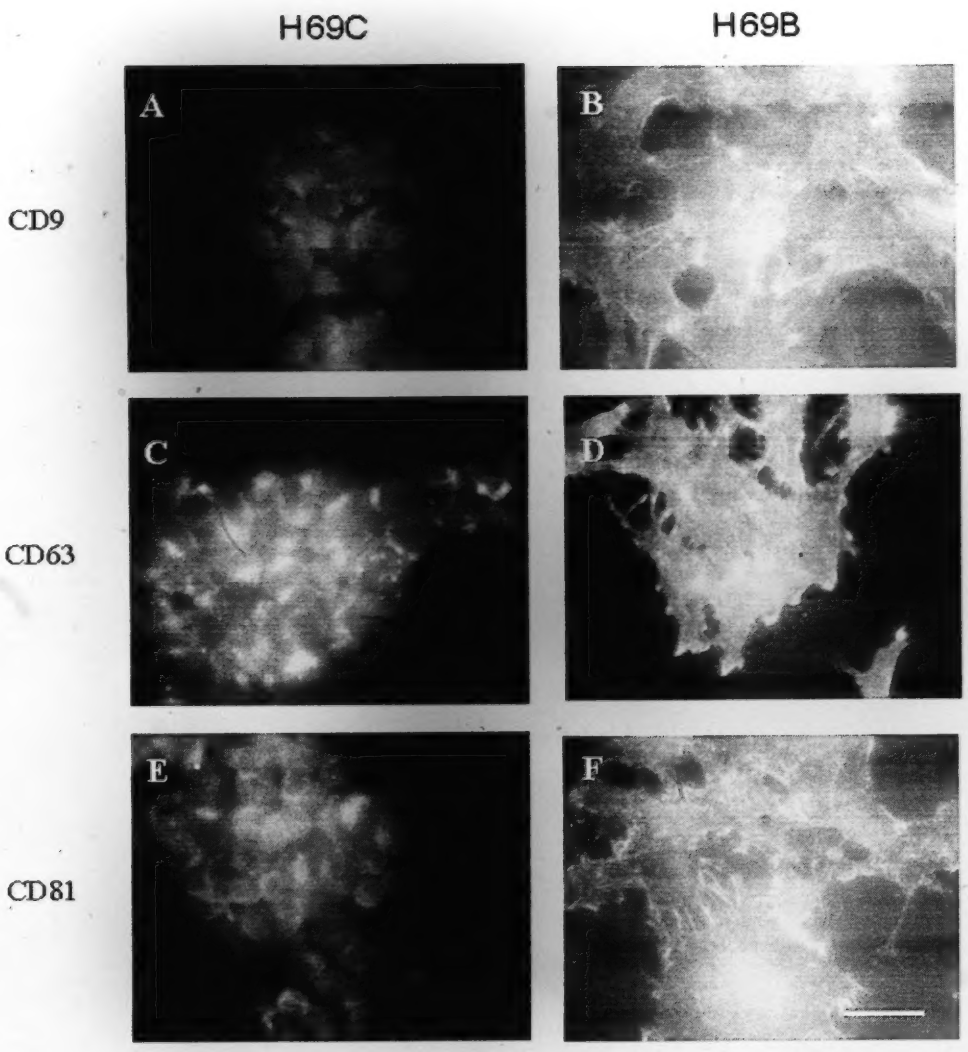


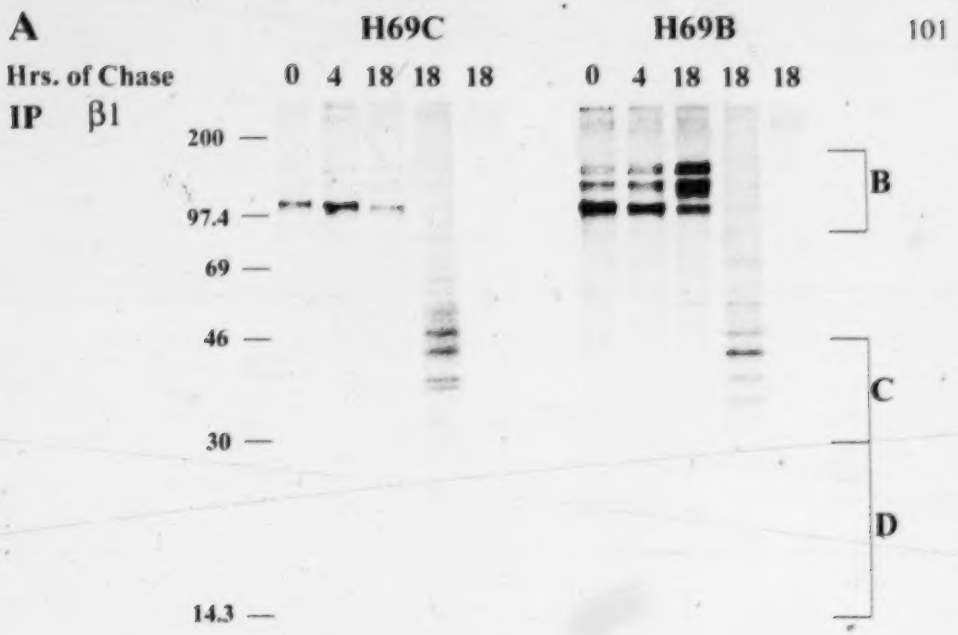
Figure 16. Re-organization of tetraspanins following conversion to an epithelial cell phenotype. Cells were processed for immunofluorescent staining as described in the legend of Fig.15, using antibodies to CD9 (A,B), CD63 (C,D) and CD81 (E,F) tetraspanins. Bar, 20 mm.

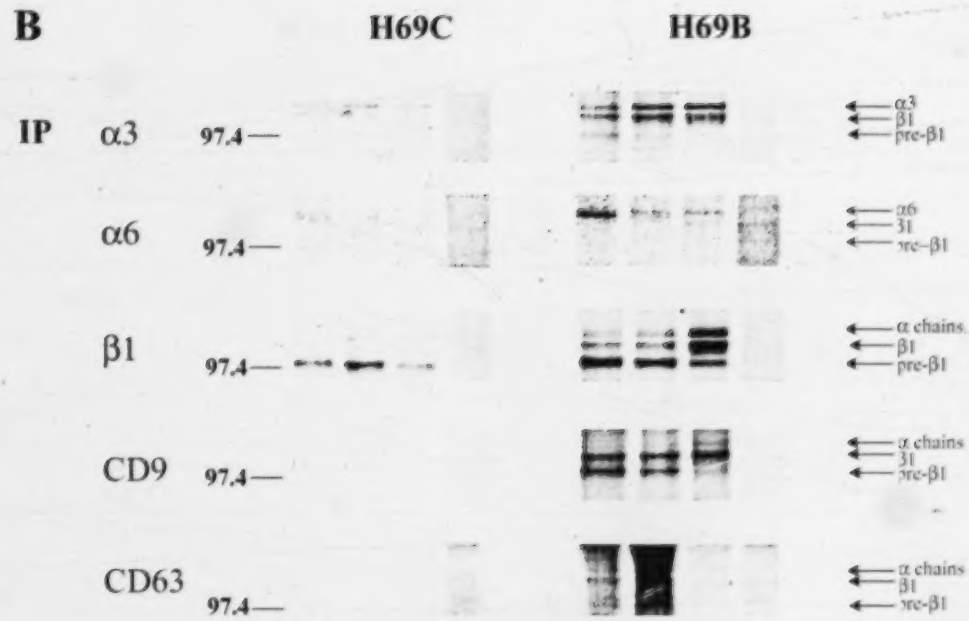
predominantly perinuclear in these cells (Fig. 16C). In H69B cells, the staining for all tetraspanins examined was increased and markedly redistributed to the cell periphery (Fig. 16B, D and F). The increased expression and redistribution of tetraspanins in H69B cells was very similar to that observed for various integrin subunits.

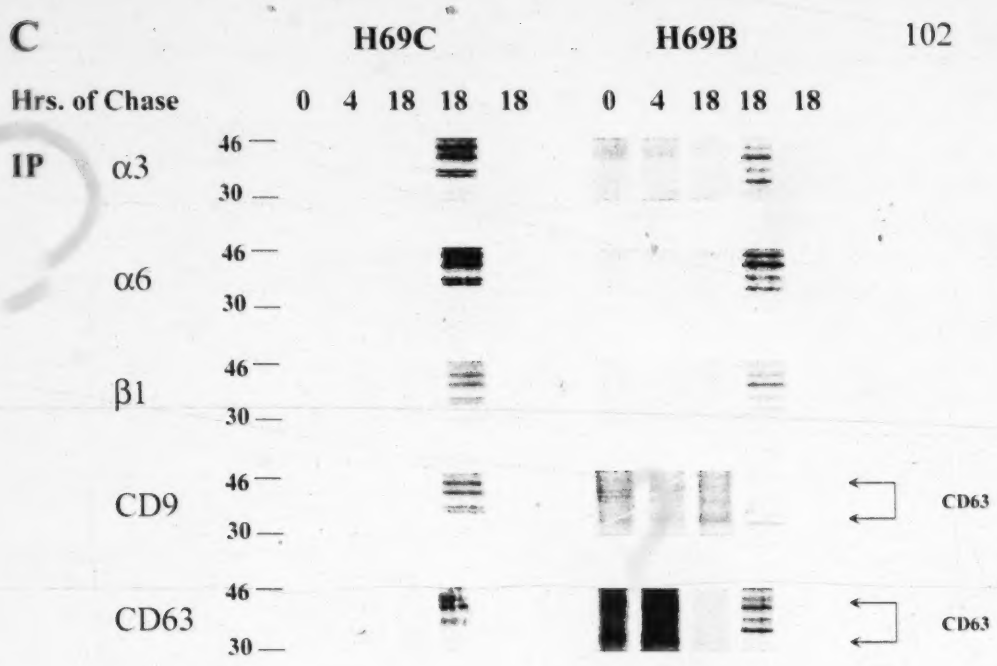
4.2.3 Synthesis, processing and turnover rates of integrin and tetraspanin molecules in H69C and H69B cells.

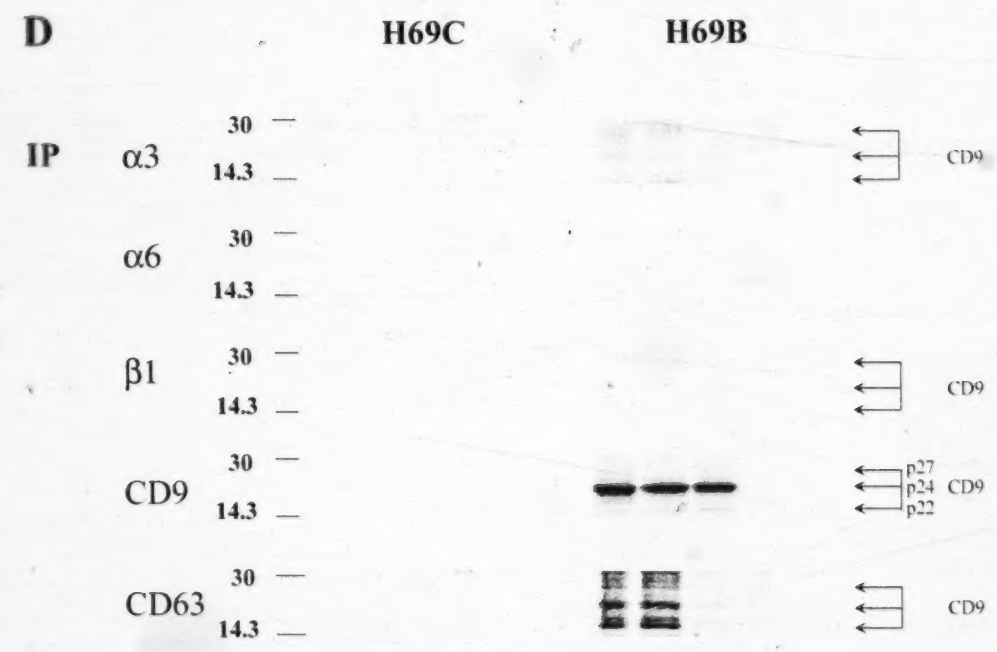
The biosynthesis and assembly of α and β_1 integrin subunits were examined in the H69C and H69B cell lines to determine if there were any differences in regulation of β1 integrin heterodimer expression. Parallel pulse chase metabolic labeling experiments were performed in H69C and H69B cells to study the maturation rates of the different integrin chains. Pulse-labeling followed by lysis in mild detergent extraction buffer allows for the isolation of protein complexes and analysis of non-covalent protein-protein interactions. To analyze $\alpha_x\beta_1$ integrin chain maturation, after 0, 4, and 18h of chase radioactively labeled cell membrane proteins were extracted in detergents under conditions that preserve integrin complex stability (295-297). Immunoprecipitation with mAbs specific to α_3, α_6 and β_1 subunits followed by separation on SDS-PAGE gels allowed visualization of co-associated integrin subunits and yielded labeled products of 140, 150 kDa and 130 kDa, respectively, under non-reducing conditions (Fig.17). Conversion of the 110/115 kDa precursor form into the fully glycosylated 130 kDa mature form of β_1 integrin results in two major bands typically observed in β_1 integrin western blots, representing isoforms that are at different stages of maturation (240). In pulse-labeled H69C cells, the β_1 subunit migrates as a lower molecular weight 110 kDa

Figure 17. Metabolic labeling and immunoprecipitation of integrins and tetraspanins from H69C (*left*) and H69B (*right*) cell lysates. After culturing for 48 hours in complete RPMI media, H69C or H69B cells were pulse labeled for 2 hours with [^{35}S]-methionine/cysteine and chased in complete medium for the times indicated. Cell lysates (*lanes 1-3 of each set*), extracted in 0.3% (w/v) CHAPS lysis buffer, were precleared with mouse serum agarose followed by Protein A Sepharose included as negative controls (*lanes 4 and 5 of each set, respectively*). Precleared lysate proteins were immunoprecipitated with mAbs against α and β1 integrin subunits, and the tetraspanins, CD9 and CD63. Immunoprecipitates were resolved in 9-15% (w/v) SDS-PAGE gradient gels under non-reducing conditions. Shown are fluorographs (A-D) with molecular weight marker positions on the left and mature/precursor (pre-β1) integrin subunits and tetraspanins positions on the right. A representative of the entire audioradiograph is shown in A; areas for each molecular weight range of interest are shown in B-D.


A
H69C
H69B
Hrs. of Chase
0 4 18 18 18
0 4 18 18 18
IP β1
200
97.4
69
46
30
14.3
B
C
D


B
H69C
H69B
IP α3
α6
β1
CD9
CD63
97.4
α3
β1
pre-β1
α6
α chains


C
H69C
H69B
Hrs. of Chase
0 4 18 18 18
0 4 18 18 18
IP
α3
α6
β1
CD9
CD63
46
30
CD63
CD63


D
H69C
H69B
IP
α3
α6
β1
CD9
CD63
30
14.3
CD9
CD9
p27
p24
p22
CD9
CD9

partially glycosylated precursor (pre- β_1) and a 130 kDa mature form under non-reducing conditions.

A representative fluorograph of metabolically-labeled and immunoprecipitated β_1 integrin subunit and β_1-associated proteins from either H69C or H69B CHAPS cellular extracts is shown in Fig.17A. The β_1 subunit immunoprecipitates from cellular extracts harvested at various time points after pulse-chase are shown. Fluorographs of radiolabeled proteins for each immunoprecipitating antibody in the 90-160 kDa range are shown in Fig.17B. Likewise, fluorographs in the range 30-46 kDa are shown in Fig.17C and from 14.3-30 kDa for each immunoprecipitating antibody are shown in Fig.17D. The kinetics of integrin and tetraspanin synthesis and turnover in H69C and H69B cells from this experiment are shown in Fig.18. Bands visualized by fluorography in Fig.17 were scanned using a scanning densitometer and readings were plotted as arbitrary units (Fig. 18A-D).

When H69C cells were immunoprecipitated with a mAb specific to integrin α_3 subunits, a band at 150 kDa, corresponding to non-reduced α_3 chains, was weakly detectable at time 0. It increased in intensity, appeared to reach a peak at 4h and was still present 18h after pulse labeling (Fig. 17B). However, this band was below the level of densitometer detection (Fig. 18A). In H69B cells, α_3 integrin chains were readily detectable at 0h and levels steadily increased from 4h to 18h (Fig. 17B and Fig. 18A). Immunoprecipitation of H69C cell lysates using a mAb against α_6 integrin chains showed a weakly detectable 140 kDa band representative of non-reduced α_6 integrin chains (Fig. 17B), below the level of densitometer detection, which appeared to decrease by 18h pulse. This 140 kDa band was of increased intensity in H69B α_6 immunoprecipitates but

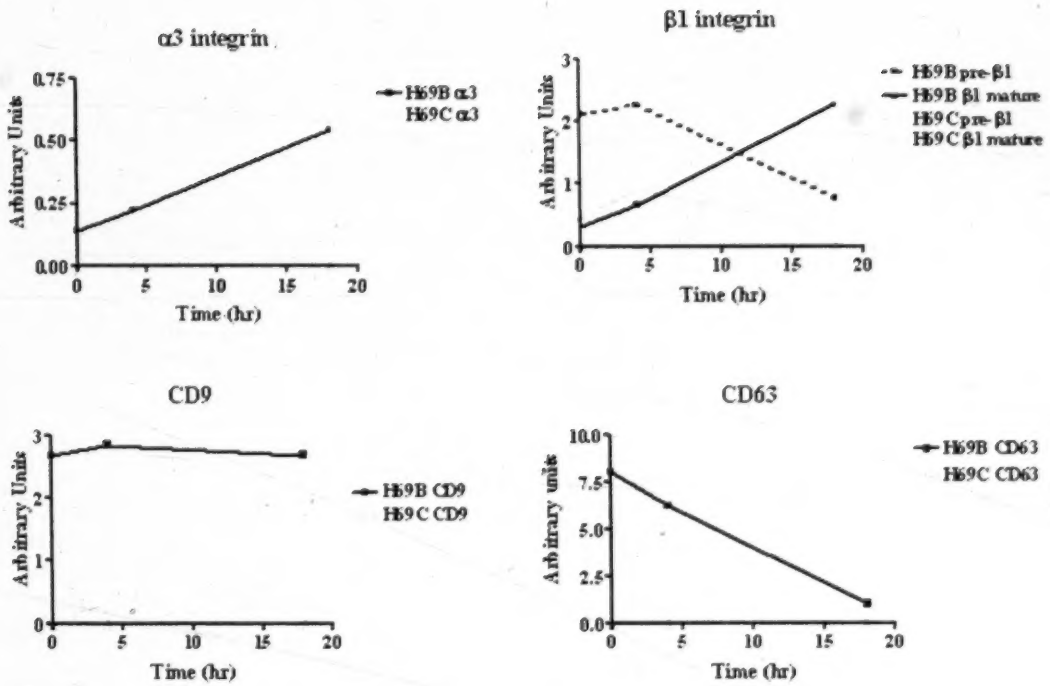


Figure 18. Preliminary analysis of the kinetics of turnover of β1 integrin subunits and tetraspanins on H69C and H69B SCLC cell lines. H69C and H69B cells were metabolically labeled and α/β1 integrin subunits and tetraspanins, CD9 and CD63, were immunoprecipitated and analysed by SDS-PAGE as described in the legend of Fig.17. Fluorographs from Fig.17 were densitometrically scanned and graphed (A-D).

also seemed to decrease by 18h. Examination of the β_1 integrin subunit immunoprecipitated from H69C and H69B cell lines revealed interesting differences (Fig. 17B). Using the 4B4 mAb (298), both pre-β_1 (100 kDa) and mature β_1 (110 kDa) could be detected under non-reducing conditions. In H69C cells, while there was a strong detectable pool of pre-β_1 chains, after only 4 hours pulse, very little mature β_1 chains appeared to be synthesized in these cells (Fig. 17B and Fig. 18B,C). At 18h 50% of this pre-β_1 integrin pool had decreased with no corresponding increase in the mature β_1 pool. In contrast, in the H69B cells, while a high level of pre-β_1 chains was evident at time 0h, mature β_1 chains were also detectable (Fig. 17B and Fig. 18B,C). After a 4-h incubation period, most of the β_1 integrin complexes in these H69B cells still contained the smaller pre-β_1 chain although in decreased amounts with a corresponding increase in the mature β_1. Unlike the H69C cells, by 18 h 50% of the pre-β_1 integrins in the H69B cells were replaced by mature β_1 containing integrins. These data indicate a half-life of pre-β_1 integrin on H69C cells of about 4-6 h under these experimental conditions whereas for H69B cells the half-life of the pre-β_1 integrins is about 12h. The half-life of mature β_1 integrin was greater than 18h for both H69C and H69B cells. Thus, the turnover rate and processing of these adhesion receptors was significantly different between H69C and H69B cells.

The half-life and processing of the tetraspanins CD9 and CD63 in these two cell lines were also investigated. Bands at a molecular weight of 24 kDa or 40-56kDa, representative of CD9 and CD63 respectively, were faintly detectable in the H69C cells (Fig. 17B and Fig. 18D,E). In contrast, in H69B cells, different forms of CD9 were

evident in anti-CD9 immunoprecipitates with very strong detection of a 24 kDa band at time 0h. The levels of CD9 seemed very stable in both H69C and H69B cells and did not change during the 18h incubation. CD63, on the other hand, seems to have a higher turnover rate in both cell lines, reaching a peak at 4h pulse and disappearing by 18h.

4.2.4 Evaluation of integrin-tetraspanin complexes in SCLC cell lines

Protein-protein associations have previously been determined to be preserved in non-ionic or weak zwitterionic detergents such as NP-40, CHAPS, digitonin, 1% Brij 96, or 1% Brij 99 (196,196,295-297,299,299). Using immunoprecipitation techniques, the addition of antigen-specific antibodies results in the isolation of associated macromolecules that specifically co-precipitate with the antigen of interest depending on protein solubility and the degree of specificity and stability of the interactions between proteins. The stringency of the immunoprecipitation conditions can be controlled by the type, concentration and hydrophobic nature of the extraction detergent used. In this regard, a requirement of mild lysis buffer to maintain tetraspanin-integrin associations has been demonstrated for various cell types (274,300). In addition, reactions performed under non-reducing conditions maintain integrin subunit associations allowing molecular weight determination of the integrin and whether the tertiary structure is required for the association. Reactions are also performed under non-reducing conditions as cysteine-cysteine disulfide bonds within the tetraspanin proteins are necessary for epitope binding of the immunoblotting antibodies.

On the basis of this approach, immunoprecipitations were performed in this study from cell lysates prepared with 1% CHAPS detergent. Associated radiolabeled proteins brought down by immunoprecipitation with antibodies specific to the protein of interest

were visualized by fluorography and identified on the basis of molecular weight (described in the previous section). Similar analysis of immunoprecipitations in non-labeled cells by immunoblotting provides specific confirmation of the identity of the associated proteins. As described in the previous section, Fig.17A shows a representative fluorograph of β_1 immunoprecipitations from pulse-labeled H69C (left) and H69B (right) cell lysates extracted in CHAPS detergent. Fig.17B examines the co-immunoprecipitation of the α and β_1 integrin subunits for each immunoprecipitating antibody as indicated, which are in the molecular weight range of 90 to 200 kDa, for both cell lines. Co-associated molecules in the molecular weight range of tetraspanins are shown in Fig.17C and D. From this data, the strength and specificity of interactions between various integrin and tetraspanin molecules are compared between the two cell types and are summarized in Table 3. In H69C cell lysates, monoclonal antibodies to α_3 weakly immunoprecipitated a non-reduced band at 150 kDa indicative of the α_3 chain, with a weakly detectable band at 110 kDa corresponding to the mature β_1 chain as well as a weakly detectable pre-β_1 band at 100 kDa (Fig. 17B). As described above, these bands are seen at all three time points (0, 4, 18h). In H69B cells however, monoclonal antibodies to α_3 immunoprecipitated a strong 150 kDa α_3 band as well as equivalent amounts of mature β_1 chain (Fig. 17B). A lesser but significant amount of the pre-β_1 in the α_3 immunoprecipitates was seen in the H69B cells which disappeared at 18h in accordance with the result observed for the anti-β_1 immunoprecipitates. In H69B cells, immunoprecipitation with mAbs against α_3 appears to bring down bands corresponding in size to CD9 (22-27kDa) (as evidenced in the anti-CD9 immunoprecipitation) which, in H69C cell α_3 immunoprecipitates, was weakly detectable (Fig. 17D). CD63, however,

Table 3 Integrin-tetraspanin associations in CHAPS detergent cell lysates

α3 IP	**H69C**	**H69B**
α3	++	++++
β1	+	++++
Pre-β1	+	+++
CD63	nd	nd
CD9	+	+++
α6 IP		
α6	++	+++
β1	+	++
Pre-β1	+	++
CD63	nd	nd
CD9	nd	+
β1 IP		
α chain	++	++++
β1	++	+++++
Pre-β1	+++	++++
CD63	nd	nd
CD9	nd	++
CD63 IP		
α chain	nd	++
β1	nd	++
Pre-β1	nd	++
CD63	+	++++
CD9	+	+++
CD9 IP		
α chain	nd	++
β1	+	+++
Pre-β1	+	+++
CD63	nd	++
CD9	nd	++++

Very strong	+++++
Strong	++++
Moderately strong	+++
Weak	++
Weakly detectable	wd
Not detectable	nd

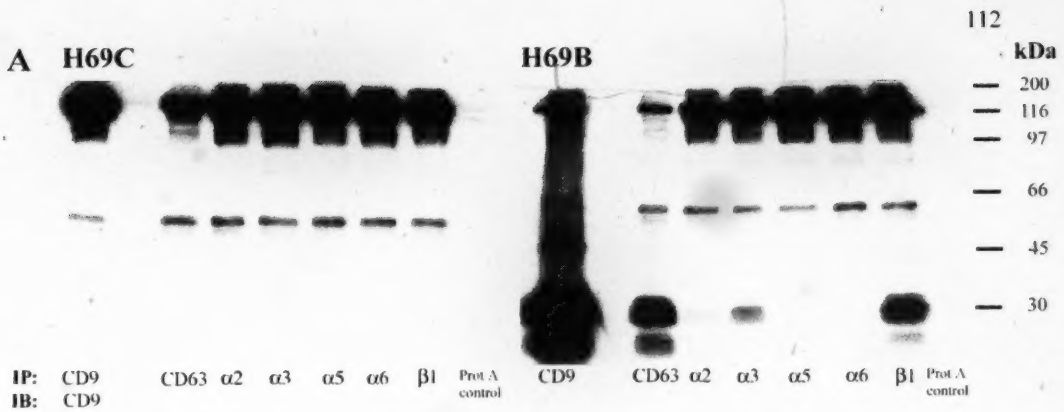
was not detectable in α_3 immunoprecipitates from either cell types. Overall, there was very little α_6 and scarcely detectable bands corresponding to the mature and pre-β_1 with no associated CD9 or CD63 in α_6 immunoprecipitates from H69C cells (Fig. 17B-D). In H69B cells, the α_6 profile showed significantly more α_6 and associated subunits than the H69C cells but was very similar in profile to that of α_3 with only weak amounts of co-precipitating CD9 and undetectable CD63. As shown in Fig.17A, mAb against β_1 immunoprecipitated multiple integrin α chain bands in the range of 140-150 kDa, a mature β_1 at 110 kDa, and a lower molecular weight band for the pre-β_1 at 100 kDa. As described in the previous section, H69C cells appeared to have a much larger pool of pre-β_1 chain in comparison to correspondingly little mature β_1. H69B cells expressed more of both pre- and mature β_1 chains than H69C cells. In contrast to H69C, H69B cells were characterized by a reduction of the pre-β_1 pool and a corresponding increase in mature β_1. In H69C cells, β_1 immunoprecipitation did not seem to co-precipitate any CD9 or CD63 although a significant amount of CD9 (but not CD63) was in β_1 immunoprecipitates from H69B cells. Suprisingly, however, in H69C cells, the reciprocal immunoprecipitation, i.e. immunoprecipitation with CD9, seemed to bring down more β_1 chain, particularly pre-β_1 chain (Fig. 17B), than even that of CD9 (Fig. 17D). Immunoprecipitation with anti-CD9 in H69B cells strongly co-precipitated both pre-β_1 and β_1 chain and more weakly, the α chain (Fig. 17B) which was similar to that of anti-β_1 immunoprecipitates. H69B cells when immunoprecipitated with anti-CD9 brought down a significant amount of CD9 (as expected) as well as co-associated CD63 (Fig. 17C-D). In CD63 immunoprecipitates from H69C cells, β_1 chains were not detectable whereas in H69B cells CD63

immunoprecipitates appear to co-precipitate pre-β_1, β_1 and α chains (Fig. 17B). On the other hand, in the reciprocal immunoprecipitation, the CD63 protein could not be easily identified in the H69B anti-α_3 or anti-α_6 immunoprecipitation (Fig. 17C). In H69C cells, CD9 appeared to be weakly detectable in CD63 immunoprecipitates (Fig. 17D). This latter association was much stronger in CD63 immunoprecipitates from H69B cells. None of the co-precipitated proteins were observed in the control mouse serum agarose/protein A preclear lanes.

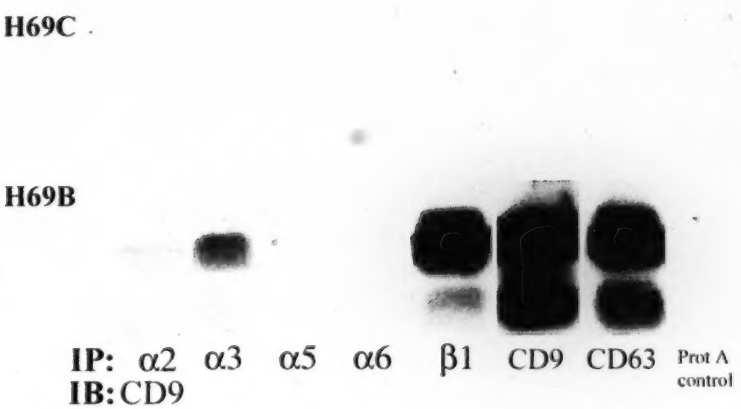
As a confirmation that the associated radiolabeled proteins, tentatively identified on the basis of molecular weight, specifically correspond to CD9 or CD63, integrin and tetraspanin immunoprecipitations from H69C and H69B cell lysates were immunoblotted with anti-CD9 or anti-CD63 antibodies (Fig. 19). A representative view of the entire immunoblot is shown in Fig.19A. Fig.19B and 19C show an enlarged view in the molecular weight range relevant to CD9 and CD63, i.e. 20-60 kDa. As there is very little CD9 expressed in H69C cells, CD9 cannot be detected in any of the H69C immunoprecipitations. However, in H69B cells, CD9 can be detected very strongly in the CD9 positive control as well as in α_3, α_6, and β_1 immunoprecipitates. In this experiment, cell lysates were also immunoprecipitated with antibodies against integrin α_2 and α_5 subunits. CD9 seemed to be weakly detectable in the α_2 but not α_5 immunoprecipitates from H69B cells. Immunoblotting of CD63 immunoprecipitates from H69C cells with anti-CD63 detected only CD63. In H69B cells, a strong band of CD63 was detectable in the CD63 immunoprecipitates with only a small amount of CD63 detectable in the CD9 immunoprecipitation. In the reciprocal immunoprecipitation in H69B cells, however, immunoprecipitation with CD63 seems to co-precipitate quite a bit of CD9.

Figure 19. Immunoblot of CD9 and CD63 tetraspanins co-precipitated with α/β1 integrin subunits in H69C (left) and H69B (right) SCLC cell lysates. Equivalent amounts of total protein from H69C and H69B cell lysates were immunoprecipitated with antibodies against various α and β_1 integrin subunits and the tetraspanins, CD9 and CD63. Immunoprecipitates were processed for immunoblotting with HRP-conjugated mAbs against either CD9 (50H.19) or CD63 (IA44). Proteins were visualized with ECL chemiluminescence. A representative of the entire audioradiograph is shown in A; an enlarged view in the molecular weight range of interest is shown in B-C.

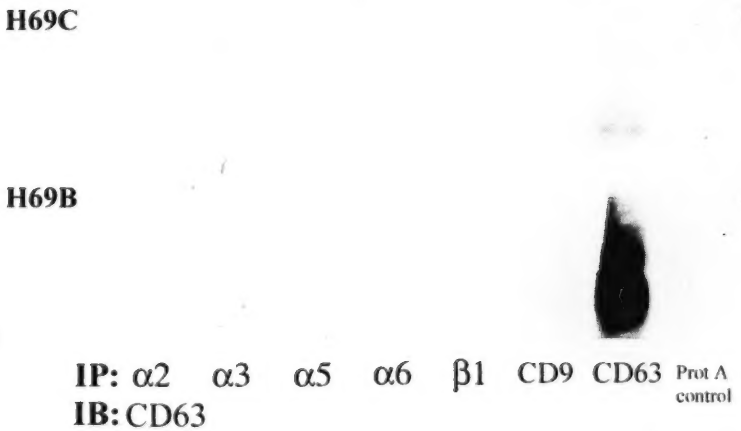
A


H69C
H69B
kDa
200
116
97
66
45
30
IP: CD9 CD63 α2 α3 α5 α6 β1 Prot A control CD9 CD63 α2 α3 α5 α6 β1 Prot A control
IB: CD9

B


H69C
H69B
IP: α2 α3 α5 α6 β1 CD9 CD63 Prot A control
IB: CD9

C


H69C
H69B
IP: α2 α3 α5 α6 β1 CD9 CD63 Prot A control
IB: CD63

4.2.5 Increased levels of integrin and tetraspanins is correlated with adhesion of H69B cells to ECM components.

To determine if the differences in levels of integrin/tetraspanin expression were reflected in functional differences of adhesion receptors, the adhesion properties of the two cell types on various defined ECM proteins were compared in a short-term serum-free adhesion assay. Previous results demonstrated that H69C adhere to laminin (301). In the present study, H69B cells were found to adhere with high efficiency to laminin and also to fibronectin and collagen (Fig. 20).

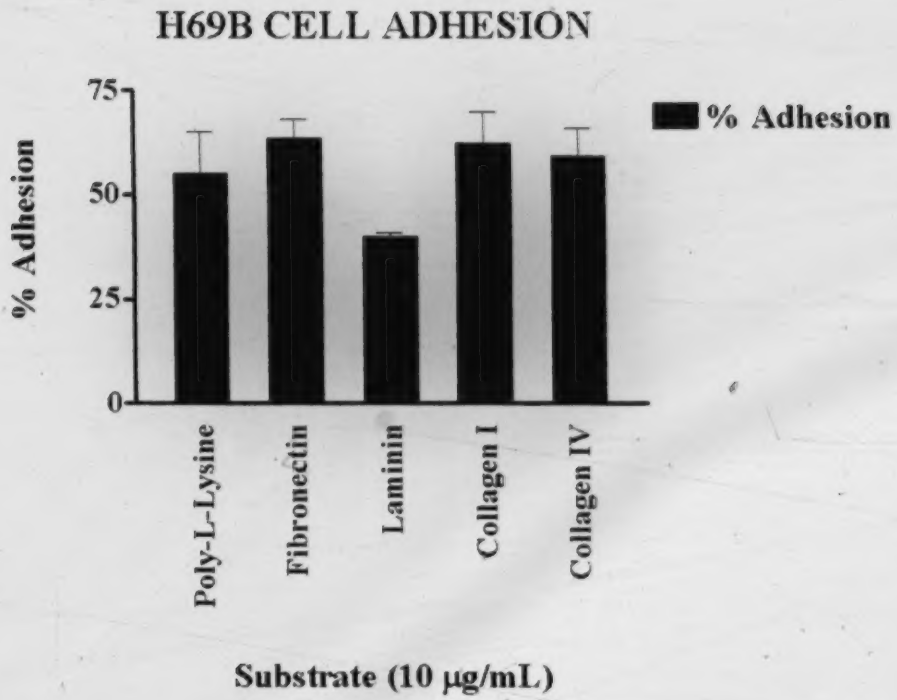


Figure 20. Adhesion of H69B cells to ECM components. Calcein-labeled cells were seeded on to microwell plates coated with the indicated substrates. After 90 minutes the plates were washed and immunofluorescence of bound cells quantitated on a MilliporeCytofluor 2350 reader. Percent binding was determined as a ratio of fluorescence of bound cells in the washed wells compared to total fluorescence of cells added in unwashed wells. Values are means of triplicate wells of two separate experiments.

4.3 Discussion

4.3.1 Altered expression of integrin cell-matrix adhesion receptors.

Epithelial-mesenchymal transitions during lung morphogenesis and differentiation are coordinated and regulated temporally and spatially by ECM adhesion receptors in response to insoluble matrix molecules and soluble growth factors (13). Changes in integrin expression and cell surface repertoire provide a way of regulating the strength and ligand preferences for cell adhesion (120). Thus, ECM composition and specificity of integrin receptors determine the proliferative and invasive properties of each cell type. Differences exist between integrin subsets in their ability to integrate physical cell-matrix interactions with intracellular signaling pathways controlling actin dynamics, cell movement, growth and survival. The synthesis of distinct integrin subsets at different stages of differentiation is one mechanism by which each cell type can control its adhesive interactions. Inappropriate alterations in integrin signaling pathways are essential for the progression of carcinomas to a metastatic phenotype.

The present study examined alterations in integrin and tetraspanin expression associated with induction of differentiation and substrate-adherence in SCLC H69 cells. Analysis of H69C cells in this experimental system shows that they have a limited integrin repertoire. Only very low to moderate levels of $\alpha_3\beta_1$ and $\alpha_6\beta_1$ were detected and these were mainly cytoplasmic in distribution. β_2, β_3, and β_4 integrin subunits were not examined in this study as previous analysis of H69C (302) and H69B cells (302) did not detect any β subunits other than β_1(219). Several studies have examined integrin expression on lung cancers, and in keeping with our results, H69 SCLC cells have been

shown to express low levels of $\alpha_3\beta_1$ and $\alpha_6\beta_1$ and no detectable expression of $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$ or $\alpha_5\beta_1$ (291-294). Transition to an epithelioid phenotype showed a striking upregulation in integrin expression in the H69B cells consistent with the development of substrate-adherence. Cell surface expression of the integrins $\alpha_2\beta_1$ and $\alpha_5\beta_1$ were highly upregulated while $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins were significantly increased relative to the levels detected in H69C cells. Immunofluoresence analysis indicated that in H69B cells $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ integrins were redistributed to the periphery of the cell, presumably to focal adhesions. Indeed, staining of the focal adhesion protein vinculin indicated a marked increase in focal adhesion complexes in H69B cells consistent with extensive cell-substrate attachment and spreading. This data suggests that the phenotypic transition is associated with alterations in integrin expression. While changes in integrin expression have been associated with carcinoma progression, expression of particular integrin subsets by specific cancer cell types have yielded conflicting reports (212). In some cases, $\alpha_5\beta_1$ overexpression resulted in a decrease in tumorigenesis (303). However, in melanoma cells, increased $\alpha_5\beta_1$ expression was correlated with an increase in invasive phenotype. These apparent contradictions likely reflect the complexity of integrin regulation and function in specific cellular contexts and in a diversity of cell types.

In addition to changes in the expression levels of integrins within a cell, the specific repertoire and activation state of integrins in a lung cancer cell compared to its normal counterpart are critical determinants of invasive and metastatic capabilities. Transient modifications of adhesion can be accomplished by 'activating' integrins without changing the levels of synthesis. In many cases, while the actual number of integrin receptors expressed on the cell surface is not altered, the activation state of a

particular integrin subset relative to another, their localization to focal contacts, and the association of various regulatory proteins may be altered. Thus, aberrant adhesion-mediated signaling pathways may occur and consequently affect cell behavior. In H69B cells, expression of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ may significantly contribute to altered integrin signaling pathways. The increase in $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins may also have functional consequences. It has been found that some integrins can modulate the function of other integrin subsets, a process known as transdominant regulation (259,304). Evidence for this was shown by $\alpha_{IIb}\beta_3$ integrin inhibition of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrin function (259). Similarly, cross-talk between $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins when both are ligated was shown in normal skin fibroblasts (260). Occupancy of the $\alpha_3\beta_1$ integrins was shown to be laminin-isoform specific and to result in a trans-dominant regulation of $\alpha_6\beta_1$ integrin clustering and formation of focal adhesions, possibly by differential triggering of one of the small GTP-binding proteins. In the present study, conversion to a differentiated phenotype led to alterations in the profile and levels of integrin expression with associated induction of *in vitro* substrate-adhesion as well as decreased tumorigenicity *in vivo*. The functional significance of induced expression of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrins as well as the effects of upregulated $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins on cellular signaling pathways and ultimately cell behavior remains to be investigated in this system.

4.3.2 Alterations in expression of integrin-associated tetraspanins

Regulation of integrin activation and function is dependent on cell-type specific expression of intracellular factors, small GTPases and associated transmembrane proteins in addition to regulation by other integrin subsets. These cellular proteins are activated in

response to both soluble and insoluble extracellular stimuli (170). Tetraspanins are a recently discovered family of membrane glycoproteins found to associate with integrins and which have been implicated as metastasis-suppressors although the basis for this effect is still unknown (178,183,195). Examination of expression levels of the tetraspanins CD9, CD63 and CD81 in H69C and H69B cells revealed changes in cell surface expression and in the levels of synthesis in association with MET transition. Of the three tetraspanins examined in H69C cells, CD63 was the only tetraspanin present in detectable amounts on the cell surface. CD63 was also distributed perinuclearly reflecting its localization to lysosomes and other intracellular vesicles (172). H69B cells showed higher levels of CD63 and detectable levels of CD9 and CD81 distributed both intracellularly and at the cell surface. Together, the modifications in the level of expression and subcellular localization of integrins and tetraspanins were associated with the development of extensive cell-substrate adhesion and formation of epithelioid monolayers in H69B cells. Expression of the tetraspanins, CD9 and CD82, have been shown to be prognostic indicators of NSCLC and to decrease tumor cell motility (191,305). In addition, transfection of CD9 into highly motile tumor cell lines has been shown to suppress motility and metastatic potential to the lung (183). The present study confirms that increased tetraspanin expression in lung cancer cells is concurrent with decreased tumorigenicity. However, other reasons for the observed reduction in tumorigenicity by the H69B cells due to other mechanisms such as enhanced apoptosis, or increased antigenicity cannot be ruled out. Interestingly, CD9 has been found to be associated with the induction of apoptosis in a Chinese Hamster Ovary cell system (306), in TCR-triggered naive T cells (307), and in the current study integrin-mediated

apoptosis might be triggered in H69B cells. Another study found that development of an SCLC adherent subpopulation actually resulted in increased invasiveness *in vitro*, although *in vivo* tumorigenicity was decreased (308). It is possible that an increase in tetraspanin expression might have the effect of making the H69B cells more migratory and invasive *in vitro* compared to H69C cells. Further studies are therefore needed to determine if there are any differences in invasion and metastasis between H69C and H69B cells and to understand how integrin/tetraspanin localization is associated with differentiation state and how this may influence cell behavior in the MET transition (see Fig. 27). It is likely that tetraspanin expression and function is important in invasion and metastasis as these proteins have been shown to be expressed in a stage-specific manner in melanoma tumors (176). The cellular localization and function of tetraspanin molecules has been postulated to modulate integrin activity (309). Ectopic expression of CD9 in the CD9-negative, poorly motile human B cell line Raji was shown to confer increased motility on fibronectin and laminin in these cells which was inhibited by both anti-CD9 and anti-β_1 integrin treatment (295). The involvement of tetraspanins in keratinocyte motility was assessed in a wound healing migration assay (309). Inhibition of cell migration was observed with antibodies to CD9, CD81, β_1, and α_3, and, to a lesser extent, to CD151. Emerging evidence from these and other studies indicate that tetraspanin-integrin complexes might be involved in transient adhesion and integrin recycling during cell migration, as well as in intercellular recognition. Other studies have implicated $\alpha_3\beta_1$-tetraspanin complexes in the control of invasive migration of tumor cells by at least two PI3K-dependent signaling mechanisms (310).

Further studies utilizing confocal microscopy will be necessary to determine if, and where integrins and tetraspanins are colocalized in H69C compared to that of H69B cells. Tetraspanins have not been reported to localize to vinculin-containing adhesion complexes (focal complexes and focal adhesions) (276). Based on antibody blocking experiments and comparative adhesion assays using cell transfectants, it does not appear that integrin-tetraspanin protein complexes regulate the attachment strength of immobile cells (276). However, the tetraspanins CD9, CD63 and CD81 have been reported to be involved in tumor cell motility and CD81/CD151 in cell migration in wound healing assays (281,311,312). The localization of integrins and tetraspanins to various peripheral adhesion structures such as the membrane extensions found in lamellopodia, filopodia and microvilli, have led to the proposal that integrin-tetraspanin protein complexes are structural components of dynamic adhesion complexes involved in modulating cell motility and invasive events (276,310,313). Tetraspanins, like integrins, have also been found in AJ at cell-cell contacts, the significance of which remains to be defined (278,281). The presence of tetraspanins on intracellular vesicles and their co-localization with integrins further indicate that they may regulate integrin subcellular localization and therefore function. Differential distribution of integrins on the cell surface is important in determining a cell's adhesive and migratory function (Fig. 27). The functional significance of two homologous integrins that recognize the same vitronectin ligand but which are distributed differentially on the cell surface has recently been characterized in terms of molecular composition of focal contacts (314). While both may function in the initial cell attachment, $\alpha_v\beta_3$ are found in focal contacts while $\alpha_v\beta_5$ integrins are not (314,315). Utilization of a combination of receptors with different affinities may be

beneficial for the versatility of SCLC cell migration (316). Evidence suggests that integrin-tetraspanin complexes may regulate the organization of the cortical cytoskeleton at the peripheral attachment sites during the extension of lamellipodia and filopodia protrusions (276,310). Preliminary examination of the localization of CD63 and CD9 staining in H69B cells seemed to indicate predominant distribution on lamellipodia (Fig. 16). Recent experiments have demonstrated the direct involvement of tetraspanin protein complexes in integrin-dependent signaling (273). Consequently, it has been suggested that tetraspanins are signaling modulators of integrin-mediated reorganization of the actin cytoskeleton possibly by modifying or intervening in integrin-associated signaling machinery (170). As discussed in section 4.1, distinct tetraspanins have been linked to protein kinase C activity. The potential for this modulation to be both negative or positive, depending on whether or not tetraspanin-associated integrins are engaged in ligand-binding and on the nature of the ligand would allow for adhesion-dependent signaling. The exact mechanisms however, remain to be elucidated. Certainly, the balance between adhesive and migratory/invasive behavior is a dynamic process, tightly regulated by ligation of cell adhesion receptors as well as cross-talk with cell-cell adhesion receptors at junctional contacts between cells.

4.3.3 Cell type transition results in alterations in maturation and processing of β1 integrin receptors.

Initial post-translational modifications of integrins and tetraspanins occur during protein synthesis in the ER. At the 4 h chase time point, in both H69C and H69B cells, there was a predominance of the pre-β_1 isoform over the mature β_1 integrin and accumulation of an intracellular pool of pre-β_1. However, analysis of the amount of β_1

subunit precursor at 0 chase showed that substantially more pre-β_1 subunit was synthesized in H69B cells during a 1h pulse with [^{35}S]-methionine/[^{35}S]-cysteine than in H69C cells. Thus, formation of the precursor β_1 integrin appeared to be accelerated in the H69B compared to the H69C. Moreover, the fate of this newly synthesized material differs markedly between H69C and the H69B cell types. In general, β_1 integrin maturation seems to vary depending on the type of cell and its activation/differentiation status. It is currently believed that the size of the intracellular pre-β_1 pool and its rate of maturation depends on the amount of α chain available (148,237,247,317,318). An excess of pre- β_1 integrin subunit seems to be essential for the maximum rate of α-subunit maturation and it is thought that this excess might guarantee that all pre-α-subunits can form a heterodimer with an existing pre- β_1 subunit. In the present study, transdifferentiation was associated with an alteration in the ratio of precursor β_1 to mature integrin isoforms in H69B cells compared to that of H69C. In H69B cells, there was a decrease in the intracellular pre-β_1 pool with a corresponding increase in mature β_1 at the 18h chase time point. In H69C cells, however, a large proportion of the β_1 precursor pool was apparently turned over before it achieved maturity (Fig. 17A,B). In the H69C cells, the decrease in pre-β1 by 18h is likely due to degradation since not much was converted to the mature β1 form and very little α chain was expressed on the cell surface. Furthermore, in H69B cells, while most of the β_1 subunit at the 1hr pulse (time 0 chase) was in the precursor form, there was already a detectable amount of mature β_1 synthesized in H69B which didn't appear until 4h chase in the H69C cells. As well, there were greater amounts of mature β_1 co-precipitating with α_3 and α_6 subunit in the H69B cells at 0 chase. This faster accumulation of mature β_1 subunit in H69B cells appears to

indicate an increase in the rate of αβ heterodimer formation compared to H69C cells. Unlike H69C, almost all the precursor β_1 was converted into the mature subunit with little evidence of degradation by 18h. In the literature, biosynthesis of the mature β_1 subunit ranges anywhere from 3h to 10h depending on the cell type and conditions analyzed. Findings by Koivista et al (247) suggest that the size of the pre- β_1 pool, the speed of maturation of integrin subunits and the receptor distribution on the cell surface may be interdependent. In their study, repression of biosynthesis of the β_1 integrin chain led to alterations in receptor maturation which had consequences for altered receptor function (247). Thus, the reduced pre- β_1 pool was seen to affect integrin maturation, localization and adhesive function. Interestingly, the size of the free pre-β_1 pool in various cell types is not stable but is regulated by growth and differentiation factors (238,247). Another study by Albiges-Rizo et al. showed that α5 integrin heterodimer maturation and glycosylation was significantly altered by reduced expression of the focal adhesion component talin (251). Analysis of H69C and H69B cell α6 immunoprecipitates at the 1hr pulse (time 0 chase) time point showed a detectable amount of the lower molecular weight α6 precursor form which was converted to mature α chain by 18h (Fig. 17B). Thus, for α6 at least, it appears that it is the processing of the β_1 chain more than α chain maturation that is affected by H69 cell transdifferentiation.

While there was a significant amount of a β_1 precursor pool in H69C cells, there was very little α chain synthesized at 1hr pulse (time 0 chase) and the amount of α chains that were expressed by the 4h time point was comparable to that of the mature β_1. H69C appeared to express limited amounts of α3 and α6 mRNA in comparison to H69B cells (Dixon, unpublished results), corresponding to the barely detectable synthesis of the α_3

and α_6 protein subunits in H69C cells. The low levels of mature α_6/β_1 subunits in H69C cells that were synthesized disappeared by 18h. Although the rate of synthesis did not seem to be altered from that of H69C cells, immunoprecipitation of α_3 and α_6 subunits in H69B cells indicated that there was an increase in the total amount of α chain synthesized. H69B cells express significantly more α_6 and β_1 subunits than H69C cells and these appear to be present in equivalent amounts by 18h. Thus, cytoplasmic pools of α_3 and α_6 integrin subunits were different between the two cell lines, in keeping with the differences observed in cell surface expression.

Immunoprecipitation with anti-α_3 showed that α_3 was co-associated with mature β1 as early as 1hr pulse (time 0 chase) in both H69C and H69B cells. In both H69C and H69B cells it seemed that all the available pre-α chain that was synthesized assembled with pre-β_1 and was transported to the Golgi. This is in keeping with previous reports that the amount and affinity of the α-chain may determine the overall maturation rate of the heterodimer. At 1hr pulse (0 chase) time point, α and mature β_1 subunits were significantly detectable at approximately equal amounts in the H69B cells even though roughly five times more pre-β_1 was present than α-chain in H69C and H69B cells. This may indicate the successful assembly of a small population of α with pre-β_1 chains immediately following pulse-labeling of H69B cells despite only a small number of complexes being assembled. In both H69C and H69B cells, $\alpha_3\beta_1$ seemed to be stabilized by 18h whereas the $\alpha_6\beta_1$ heterodimer appeared to decrease by 18h. As there was no evidence of β_4 chains, which can also heterodimerize with the α_6 integrin subunit, it appeared that all of the α_6 was $\alpha_6\beta$1. The higher expression levels of newly synthesized $\alpha_3\beta_1$ over $\alpha_6\beta_1$ was consistent with cell surface expression as determined by flow

cytometry. However, in keeping with less α chains in H69C cells, the maturation of integrin heterodimers appeared limited. In H69B cells, in contrast, there seemed to be no deficiency in the maturation rate of β1 integrins and the association of mature α and β_1 integrin chains for both $\alpha_3\beta1$ and $\alpha_6\beta1$ by 18h indicated that the majority of $\alpha\beta_1$ integrin heterodimers were assembled by 18h. How many of these differences between H69C and H69B cells were due to direct increases in α subunit expression in H69B cells versus maturational deficiencies of integrin heterodimers in the H69C cells remains to be determined.

Following assembly in the ER and transport to the Golgi, subsequent processing in both H69C and H69B cells appeared to occur rapidly as mature β_1 chains were detected even as early as 1hr pulse (time 0 chase). In both cell types there was an immediate appearance, although at low levels, of α_3 and mature β1 even by 1hr pulse (time 0 chase). The rate of synthesis of the mature β_1 integrin chain paralleled that of the α_3 and α_6 subunits for both cell lines although in H69B cells it seemed there was an equivalent amount of mature β_1 subunit with that of the α chains. While 18h was adequate to determine the turnover rate of precursor β_1 integrin, further studies with longer or shorter cell labeling and chase times will be necessary in order to determine the stability/ $t_{1/2}$ / turnover rate of the α and β_1 integrin chains in H69C and H69B cells. This will determine if any differences in processing of β_1 integrins between the two cell type results from differences in mature β_1 stability. Thus, transdifferentiation may be associated with increased α chain synthesis and an increased maturation rate for β_1 integrin heterodimers consistent with their increased cell surface expression.

Multiple glycosyltransferases in the Golgi are responsible for the final steps in processing integrin oligosaccharides. Biosynthesis of the β_1 integrin is associated with an increased activity in these processing enzymes in Ras-transformed NIH-3T3 fibroblasts (238). Changes in patterns of integrin glyosylation have been seen in transformed and cancer cells (319). Specifically, Ras oncogene activation is associated with altered pre-β_1 and mature β_1 expression, maturation and glycosylation (238). Modulation of integrin maturation is a mechanism that is beginning to be elucidated. In osteosarcoma and epithelial cells, the growth factor TGF-β1 has been shown to modifiy β_1 integrin processing thereby altering integrin surface expression and affinity of cells for particular ECM ligands (239). In fact, it was shown that Ras mediates two stages in β_1-chain maturation: a TGF-β1 independent process and a TGF-β1 mediated conversion of the 115 kDa β_1 integrin precursor into the fully glycosylated 130 kDa mature form and that this conversion takes place in the Golgi (239). As a result of a dominant negative Ras mutation, HD3 colon epithelial cells exhibited altered glycosylation of the integrin β_1-chain, decreased cell surface expression of the mature β_1 integrin, and impaired binding to collagen and laminin (239). Given the role of Ras and TGF-β1 in SCLC pathogenesis, the significance of autocrine/paracrine TGF-β1 regulation of β_1 integrin maturation in SCLC in terms of adhesion and invasive function needs to be examined. Further studies using treatment of cells with tunicamycin, to block N-glycosylation, and treatment with Endo H would determine if the glycosylation of the β_1 integrin is altered between the two cell types. Sensitivity to Endo H, which can remove high mannose but not complex N-linked oligosaccharides from glycoproteins, would indicate whether high mannose oligosaccharides are being converted into complex forms in the Golgi. Alternate

glycoforms of mature β_1 integrins correlates with profound alterations in cell phenotype. Deficits in cell adhesion results not only from diminished cell surface expression of integrin heterodimers, but also from reduced affinity of aberrantly glycosylated integrin isoforms for ECM ligands (250,256). Whether N-glycosylation of β_1 integrins is blocked in H69C resulting in decreased surface expresssion and altered cell attachment to ECM needs to be determined. Whether the deficiency in β_1 maturation is at the level of gene expression, stability or processing remains to be seen in H69C cells. Determining the differences in integrin processing between the parental and differentiated cell lines in this model system will further our understanding of how pathways that regulate integrin processing and maturation contribute to integrin adhesive, invasive and signaling function.

4.3.4 Alterations in tetraspanin synthesis, processing and turnover are associated with transdifferentiation in SCLC cells

Tetraspanins have four highly conserved cysteines in the transmembrane region and can be labeled with [^{35}S]-cysteine. Very little CD63 was synthesized in H69C cells (Fig. 17C; CD63). In contrast, in H69B cells there was a rapid synthesis of CD63. CD63 is an integral membrane protein consisting of a 23.5 kDa core protein that is modified to various degrees by N-glycosylation. The CD63 molecule possesses three sites of N-glycosylation (320) that leads to a precursor form with a molecular weight of approximately 34 kDa (188). Further processing of CD63 results in a heterogeneity of the apparent molecular mass ranging between 29 and 70 kDa (188). In H69C and H69B cells, at 1hr pulse (time 0 chase) there was the rapid appearance of an electrophoretic smear of glycosylated forms of CD63 in the range of 29-70 kDa. Incubation of cells with an

inhibitor of protein N-glycosylation, such as tunicamycin, allows for the determination of the amount of the CD63 core polypeptide synthesized (188). In both H69C and H69B cells, CD63 synthesis appeared to reach a peak by 4h although CD63 levels were decreased by 18h. The turnover of CD63 in G361 melanoma cells has been shown to have a half-life between 22 and 46 h (188). Interestingly, treatment of human melanoma G361 cells with the phorbol ester PMA resulted in apparent partial inhibition of CD63 glycosylation. Treatment of G361 cells with both PMA and protein kinase C inhibitors re-established the usual CD63 glycosylation pattern indicating that the observed effect of PMA on CD63 glycosylation was reversible and mediated by protein kinase C activation (321). The significance of post-translational modifications such as glycosylation, myristoylation and acetylation and the role and nature of tetraspanin function is only beginning to be elucidated (Fig. 27). A recent study found that complete glycosylation of CD9 and CD82 was essential for haptotactic and phagokinetic motility and apoptosis-inducing ability in CHO cell transfectants (306). Interestingly, in SCLC, increased expression levels of glycosyltransferases and gangliosides resulted in enhanced cell proliferation, and apoptosis was induced by an anti-glycolipid mAb against the ganglioside GD2 although this mechanism remains to be identified (322). Other evidence of a regulatory role for glycosylation sites in tetraspanin function was suggested when a feline CD9, which lacks a predicted glycosylation site found in human CD9, was expressed in a human B cell line and appeared to have functional consequences on the motility response of these cells on a fibronectin substrate (323). Thus in addition to regulating integrin function, glycosylation may be a mechanism by which tetraspanin function is also modulated (Fig. 27).

Whereas CD9 expression was not even detectable in H69C cells, in H69B cells CD9 was synthesized as several isoforms. In platelets and other cell types, the mAb 50H.19 immunoprecipitates CD9 glycoproteins of 22, 24 and 27 kDa which have been shown to be due to differences in glycosylation and appear to be tissue specific (324). The core 22 kDa band is O-glycosylated, whereas the 24 kDa has additional N-glycosylation. It is possible that there are tissue-specific variants of CD9. Like CD63, CD9 was rapidly synthesized in H69B cells, at a faster rate than that of the integrins, as a significant proportion of CD9 was labeled by 1hr pulse (time 0 chase) (Fig. 17D; CD9). It is possible that an available pool of tetraspanins is required in order for them to act as integrin chaperones.

4.3.5 Changes in integrin-tetraspanin complexes

As the functional role of tetraspanins is as yet unknown, the significance of their upregulation in H69B cells is not immediately apparent. Since it is believed that tetraspanins may exert a role in regulating cell proliferation and migration through their interactions with specific integrins (172,276), these associations were characterized and examined for differences between the two cell types. In H69B cells, it appeared that CD63 bands in the range of 30-60 kDa were associated with α_3 and β_1 subunits (Fig. 17C). Corresponding bands at the molecular weight of CD63 were not seen in either the mouse serum agarose or the protein A preclear controls indicating that the co-association is specific and not due to non-specific binding. Future studies could utilize Endo F to remove complex carbohydrates and would reveal a sharper band that would specifically clarify and confirm the identity of CD63 in these immunoprecipitates. CD63 did not seem

to be present in the α_6 immunoprecipitation or was below the level of detection, further confirming that co- immunoprecipitation with α_3 is a specific association. Whether CD63 was interacting directly with the α_3 integrin or indirectly through association with CD9 has not been determined.

CD63 is also co-associated with CD9 since very strong bands characteristic of several CD9 isoforms were visible in the CD63 immunoprecipitates. Curiously, in H69C cells, there seemed to be more CD9 in the CD63 immunoprecipitation than in the CD9 immunoprecipitation, although only the lower molecular weight 22/24 kDa CD9 isoforms appeared to be co-associating. Berditchevski et al have shown that assembly of the $\alpha_3\beta_1$-CD151 tetraspanin complex is an early step during integrin biosynthesis ie. 15 minutes from the onset of labelling, whereas the co-immunoprecipitation of $\alpha_3\beta_1$ with CD9, CD63 and CD81 is seen significantly later (at 45 minutes of chase) (325). As CD151 (but not other tetraspanins) could be co-immunoprecipitated with the non-cleaved form of the α_3 subunit, the initial interaction between the proteins should occur in the ER or in the pre-Golgi compartment before pro-α_3 cleavage in the Golgi. Their observations supports the idea that the assembly of $\alpha_3\beta_1$-CD151 complexes take place before $\alpha_3\beta_1$ reaches the cell surface and that the pre- α_3 subunit is associated with CD151 before that of the β_1 subunit. Whether assembly of α_3-CD151 intermediates in the ER offers an alternative to that of calnexin for the recruitment of the β_1-subunit into the complex remains to be investigated. CD9 associates with calnexin independently of its association with the β_1 integrin, suggesting that calnexin could be involved in the processing of CD9 (240). CD9 association with pre-β_1 integrins has been shown in a study by Rubinstein et al (240). This association was detected as early as 15 min after metabolic labeling, and the use of a

Golgi function inhibitor, Brefeldin A, demonstrated that the formation of CD9-pre-β_1 complexes does not require Golgi modifications of either CD9 or integrin β_1. They demonstrated the specificity of this interaction by the fact that other tetraspanins, CD63, CD81, and CD82, did not associate with pre-β_1 (240). In the present work, it is unclear as to whether pre-β_1 in either H69C or H69B cells is associated with CD63, although the association of pre-β_1 with CD9 is very strong.

Small amounts of 22, 24 and 27 kDa isoforms of CD9 were seen in the α_3 immunoprecipitation of H69C cells. These bands were not evident in the mouse serum agarose or protein A controls. Suprisingly, only the lower molecular weight isoforms appeared to be co-precipitating in all three time points and there seemed to be even more CD9 co-precipitating with α_3 than immunoprecipitated with the anti-CD9 antibody. This may be due to differences between monoclonal antibodies in their ability to recognize conformational epitopes that may be masked or altered in the process of assembling protein-protein complexes. In H69B cells, appreciable amounts of CD9 were co-precipitated with both α_3 and β_1 even at the 1hr pulse (0 chase) time point while CD9 co-precipitation with α_6 was undetectable. The amount of CD9 associated with $\alpha_3\beta_1$ was not enhanced by the presence of increased levels of mature integrin heterodimers. This may suggest that there is a saturable upper limit for tetraspanin involvment in the integrin maturation pathway. For both H69C and H69B cells, confirmation of the presence of CD9 in α_3, α_6, β_1 and CD63 immunoprecipitates, in addition to those of α_2 and α_5, was analyzed by immunoblotting. In these experiments, only CD63 was present in the CD63 immunoprecipitate and also faintly in the CD9 immunoprecipitate in H69B cells. This

immunoprecipitation/ immunoblotting experiment was repeated twice and the faint bands in the α_2 and α_6 were detected both times.

It is unknown as yet how an increase in integrin-tetraspanin complexes in H69B cells could possibly alter integrin signaling pathways since the biochemical function of tetraspanins remains to be elucidated. Numerous studies have reported the association of $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrin receptors with CD9 and CD63 to form complexes at the cell surface and these are localized to lamellopodia at the leading edge of migrating cells (170,296,297,310). Given the role of tetraspanins in membrane fusion, tetraspanin-specific constitutive association with PI4K, the presence of CD63 on intracellular vesicles and the ability of tetraspanins to self-associate, it is possible that tetraspanins aid in the regulation of integrin trafficking (155,326). In addition to membrane trafficking events, the importance of phosphoinositide signaling in regulation of cytoskeletal reorganization and assembly of specific focal contacts is starting to emerge (Fig. 27) (139,252). While integrin clustering can be induced by binding to multivalent ligands (an outside-in generated stimulus), integrin clustering *in vivo* may also be regulated by intracellular signaling events in response to growth factors/ chemoattractants. Such a stimulus could, for example by altering the lipid and protein composition of membrane microdomains, influence the lateral diffusion rates and the accumulation and organization of integrins into specific microdomains within the cell membrane. While the functional significance of tetraspanin-integrin associations is still not established, the complexity of these interactions and functional relevance in defining cell behavior suggests that integrin function may be modulated at many levels. It is likely that tetraspanins, by their ability to co-associate and form a tetraspanin web, influence function-specific integrin clustering

perhaps by determining their participation in, or exclusion from, signaling-specific lipid raft domains (274,327). This determination of integrin localization may also affect integrin signaling function in both a ligand-dependent and ligand-independent manner (278). Thus, the combinations of different integrin conformational states and associations to various tetraspanins might contribute to the generation of differential signaling complexes, both temporally and spatially (Fig. 27). Distinct integrin receptor functions can arise from three types of transmembrane signal: ligand occupancy, receptor aggregation, or both together (117). Ligand occupancy may be enough to generate integrin conformation-dependent association and activation of cytoskeletal and signaling molecules, such as tensin and FAK (see section 1.3.4.2) to obtain formation of initial focal complexes. With activation of signaling enzymes such as PI kinases, PKC, and FAK as well as small GTPases, these transient structures can disappear or evolve to focal adhesions (Fig. 27) (156,328). Integrin-mediated assembly of focal adhesions and cytoskeletal reorganization, however, requires both ligand occupancy and integrin aggregation in order to obtain stabilized integrin-ligand interactions required for cell spreading and firm adhesion (139).

4.3.6 Transdifferentiation of SCLC cells is concurrent with altered cell-matrix adhesion.

As H69B cells express α_2, α_3, α_5 and α_6 subunits, we wanted to examine the functional specificity of integrin adhesion receptors on ECM proteins resulting from transdifferentiation. Functional differences in substrate-adherence of H69B cells were compared to the adhesion profile of H69C cells. H69C cells are non-adherent in vitro, therefore, activation of integrins was stimulated using Mn^{2+} before determining

functional adhesive status to various matrix components (301,302). In keeping with the expression of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrin receptors, H69B cells exhibited a wider binding profile to matrix components. The integrin $\alpha_3\beta_1$ functions as a receptor for laminin, collagens, and as a broad specificity fibronectin receptor (Fig. 6A) (104,329). The integrin $\alpha_6\beta_1$ is a laminin receptor. Therefore, differences in substrate adhesion reflects the difference seen in integrin expression between the two cell types and is consistent with the possibility that the observed integrin-tetraspanin associations reflect the differences in expression and distribution seen between the two cell types. Previous studies have determined adhesion specificity in normal lung bronchial epithelial cells as well as lung cancers (3,293,330). Studies show that normal lung cells express $\alpha_3\beta_1$ and $\alpha_6\beta_1$ collagen and laminin receptors (3,331).

Significantly, upregulation of the cell adhesive complexes in this experimental system coincided with decreased tumorigenicity as the adherent H69B cells were apparently completely non-tumorigenic. Given the observed increase in cell-cell and cell-matrix adhesion in H69B cells and a transition to a more epitheloid phenotype, it is likely that these changes have implications for the process of metastasis. One study utilizing a SCLC cell line demonstrated a direct link between decreased $\alpha_3\beta_1$ integrin expression and increased tumorigenic behavior as a consequence of *c-myc* transfection and overexpression (332). Also, a link between substrate adhesion and decreased invasiveness in SCLC has been suggested by Khan et al. (308). These investigators found that adherent lines isolated from non-adherent parental cells exhibited a decrease in neuroendocrine markers suggestive of a shift in phenotype from SCLC to non-SCLC-like concurrent with a reduction in their metastatic potential (308). Thus, cell-substratum interactions can have

a profound effect on the differentiation status of these cells. The expression of oncogenes such as *myc* and *ras*, together with cell aggregation and substrate attachment modulate transitions between SCLC and the more adherent non-SCLC phenotypes along a differentiation continuum (333). Further understanding of the specific mechanisms in terms of integrin signaling and tetraspanin regulatory function, may allow the manipulation of these pathways in the development of effective strategies in the treatment of SCLC.

CHAPTER 5 GROWTH, DEATH AND SURVIVAL PATHWAYS IN THE SCLC MODEL SYSTEM[1]

5.1 Introduction

Differentiation, growth and apoptosis are alternative cellular pathways, each crucial for developmental control and establishment of tissue-specific functions (136). Most normal cells depend on environment-specific factors such as growth factors and ECM supplied by neighboring cells to maintain their viability. Bidirectional communication between a cell and its microenvironment is dependent on information relayed and integrated by cell surface adhesion receptors. Indeed, intercellular contacts and cell-matrix interactions exert major effects on phenotypic features such as gene regulation, cytoskeletal structure, differentiation and growth control. Current insight into adhesion signaling pathways reveals that they converge on the same pathways with those identified in studies of oncogenes and growth factors. Identification of tyrosine kinases, small GTPases, and lipid mediators activated downstream of integrin signaling has led to the realization that common mediators such as c-Src, phosphinositides, and PKC are activated by both growth factor and adhesion stimulation. While individually the degree of activation and contribution of each pathway is low, the combined output of both is synergistic (165). This coordination in signaling between soluble factors and adhesion to cellular matrix proteins is particularly significant in the influence of adhesion on cell growth and survival.

[1]R. Meuser contributed to Fig. 24.

5.1.1 Converging growth factor and integrin signaling pathways

Integrin regulation of cell cycle progression is just beginning to be characterized. Integrins regulate cell proliferation through activation of multiple pathways (134,165). As a molecule at the crossroads between converging growth factor and integrin signaling pathways, FAK has been found to have a pivotal role in linking the two pathways. Through FAK, integrin signaling is linked to ERK activation (134). Activation of ERK allows progression through the G_1 phase of the cell cycle in response to soluble mitogens. While growth factors induce a strong, acute ERK activation, they are not sufficient to overcome the threshold necessary for cell cycle progression. Sustained ERK activation requires an association of growth factors with integrin adhesion receptors (334). Integrin ligation induces a milder activation of ERK with slower kinetics and lowers its threshold. Integrin-mediated adhesion to the ECM combined with growth factor signaling, permits efficient activation of ERKs (298). It is now believed that the threshold for permissive growth or migration may be conditionally dependent on, and restricted by, a requirement for redundancy and convergence between numerous signaling pathways. Besides activating the Shc/Grb2/Sos pathway, outlined in section 1.3.4.2 and Fig.7, an additional means by which FAK can activate ERK is through recruitment of the docking molecule p130Cas (335). Regulation of the phosphorylation and activation state of p130Cas by the FAK-Src kinase complex facilitates further activation of multiple tyrosine kinase, phosphatase and adaptor signaling pathways. Like Shc, p130Cas also binds to Grb2/Sos leading to activation of the Ras/MAPK pathway. In addition, p130Cas binds directly to C3G, a GEF for the small GTPases Rap-1 and R-Ras has been shown to activate the Jun N-terminal kinase (JNK) pathway (Fig. 7). This family of MAPK kinases activates

growth-regulatory transcription factors. Autophosphorylated FAK also binds to phosphoinositide-3-OH kinase (PI3-K) linking integrins with the anti-apoptotic protein kinase B/ Akt pathway (137). Activation of FAK alone is still not enough to promote Ras-ERK signaling in normal cells. Distinct ECM signals are mediated through specific ligation of integrins capable of directly recruiting the adaptor protein Shc. Ligation of specific integrin receptors, in association with the scaffolding protein caveolin-1, results in recruitment of Shc by the integrin α subunit, and Shc phosphorylation. Unlike FAK and p130Cas, which contribute to the late phase of integrin-dependent activation of ERK, Shc mediates the early and peak phase of ERK activation (137). Thus, Shc and FAK pathways are activated in an independent and parallel manner. As a result, ERK activation is regulated not only in amplitude but also temporally. Integrins can also activate the JNK signaling pathway through Rac activation and the potential requirement for both ERK and JNK activation may be key inputs needed to induce expression of early-intermediate genes and progression through the G_1 phase of the cell cycle (137,165). The ECM microenvironment ultimately determines cell behavior, however. For example, ligation of integrins not capable of recruiting Shc results in exit from the cell cycle and apototic cell death, even in the presence of mitogenic concentrations of soluble growth factors (336).

5.1.2 Significance of anchorage dependent behavior of cells

Convergence of integrin signaling pathways with mitogenic growth factor pathways ensures the anchorage dependence of cells upon attachment to substrate for continued cell survival (159,334). Cells that are unattached to basement membrane or

ECM proteins, or that are attached with inadequate or inappropriate cell-matrix contacts undergo an active form of apoptosis referred to as 'anoikis' (205). This may serve to prevent cells that are displaced from their normal environment, not only from growing but also from surviving in non-physiological sites. Thus, in response to cellular stimuli, cross-talk between integrin signaling and cellular pathways involved in the delivery of proliferative, pro-apoptotic, cell-specific differentiation or migratory signals provides tight regulatory control in the specificity of cell function. Tumor cells, however, are capable of growing without attachment to substratum, a property that has been called anchorage independent growth (337). Mutations in growth factors and/or receptors may be mitogenic but not necessarily oncogenic. Thus, combined anchorage independence and growth factor/serum independence are requirements for tumor cell survival and growth. This ability of tumor cells confers an advantage necessary for their inappropriate localization *in vivo*, as occurs in invasion and metastasis (338). Tumor progression, then, is a function of both enhanced cell proliferation and aberrant cell survival resulting from suppression of apoptosis.

5.1.3 Cell death mechanisms

In normal cell and tissue function, apoptosis is a mechanism of physiological cell death in response to environmental and developmental signals. During remodeling of tissues after injury or involution and maintenance of cell number and tissue architecture, the process of apoptosis eliminates unneeded, mislocated or damaged cells. Unlike necrotic cell death, apoptosis is an active process characterized by cell shrinkage, nuclear condensation, and membrane blebbing. Apoptotic cells are efficiently executed, packaged

and disposed of by phagocytosis prior to releasing their intracellular contents, potentially avoiding an inflammatory reaction (339). Many of the proteins in the apoptotic machinery are constitutively expressed so that when a cell is deprived of signals from other cells or fails to obtain the extracellular signals needed to suppress apoptosis, the death programme operates by default (340). Balance between apoptosis and cell survival in maintaining tissue function is regulated by either death inhibitor or death promoter families of related proteins. Within these families, regions of homology called death domains mediate protein-protein interactions that determine their heterodimerization, affinities for one another and cellular localization. Such interactions influence the biochemical equilibrium between survival and death (341). The most well-characterized family of anti-apoptotic proteins, Bcl-2, acts to enhance a cell's capacity to survive under a variety of sub-optimal conditions or cytotoxic insults. Bcl-2 blocks cell death by preventing the activation and mitochondrial release of caspase-9, an initiator in the activation of a cascade of executioner proteinases, which otherwise would consign the cell to apoptotic death. Unfortunately, pathological elevation in Bcl-2 expression confers apoptotic resistance associated with a cancerous state. In SCLC tumors, significant expression of Bcl-2 was detected both by Northern (mRNA) and Western (protein) blotting, but this was not seen in the regions of adjacent normal lung examined (342). Bcl-2 protein has also been found in NSCLC and some squamous carcinomas of the lung (8). Another study looked at expression of apoptosis-related factors p53, Bcl-2 and the pro-apoptotic factor, Bax, in neuroendocrine lung tumors (343). In high-grade NSCLC and SCLC tumors, the authors found a correlation between Bcl-2 overexpression and Bax down-regulation that correlated with a lower apoptotic index. Paradoxically, another

study found that Bcl-2 expression was associated with increased apoptotic index (15). However, as tumor cells which have inactivated the apoptotic machinery upstream of Bcl-2 no longer require Bcl-2 expression, tumor cell response to apoptotic stimuli may be more accurately determined by examining the ratios of Bcl-2 members rather than expression of any one protein (344).

In addition to these pro- and anti-apoptotic factors, the apoptotic machinery consists of adaptor proteins containing interacting death domains, which link signaling events to caspase activation (339). Activated caspases (cysteine proteinases with specificity for aspartic acid residues) are the arm of the apoptotic machinery, mediating the execution phase of cell death. Proteolytic cleavage of the inactive caspase proforms found throughout the cell, by "initiator" caspases, subsequently generates a cascade of activation and cleavage of "executioner" caspases. These target a wide variety of proteins and key substrates in cell function. For example, the action of caspase-activated DNase is responsible for the oligonucleosomal DNA degradation observed in cells undergoing apoptosis. This final phase of DNA degradation prevents oncogenic transformation and acts as a mechanism of viral defense. The activation of endogenous nucleases on a preserved chromatin structure generates a ladder of DNA degradation that is a hallmark of apoptosis in vitro (345). Other changes characteristic of apoptosis, measurable *in vitro*, include: loss of membrane integrity, nuclear compaction, cytoplasmic condensation, disintegration into dense particles and translocation of phosphatidylserine in the plasma membrane (345).

Dysregulation of intrinsic cell proliferation and death pathways is therefore a determining factor leading to clonal expansion and tumor growth. As described in

Chapters 3 and 4, our SCLC experimental system was associated with reduced tumorigenicity in the H69B cells that had undergone upregulation of cell-cell and cell-matrix adhesion receptors to a more adhesive and differentiated phenotype. Thus, we wanted to further examine these cells, to determine if pathways regulating growth, anchorage dependence, sensitivity to apoptotic mechanisms and cell survival were also altered compared to that of the parental H69C cells. The experiments described in this chapter were based on four considerations: 1) to compare and evaluate differences in growth rate between the two cell types to see if they reflected the changes in morphological and adhesive status associated with differentiation. 2) to determine the anchorage dependency of the two lines. 3) to determine susceptibility to apoptosis in the two cell lines and 4) to compare and evaluate whether upregulation of integrins and tetraspanins in the transition to an adherent phenotype in the H69B cells contributed to a dependence on attachment to ECM proteins for survival compared to that of the H69C cells.

5.2 Results

5.2.1 Growth of derivative cell lines

The growth curve of the adherent H69B cells was assessed to determine if the changes observed in morphology were associated with changes in growth. The growth of the parental H69C cells has doubling times around 24h. Manual analysis of H69B growth using a hemocytometer to count cells, and also using an MTT proliferation assay, showed that H69B has a doubling time between 4-5 days in culture. This is decreased, in comparison to the parental H69C cells, although similar to that observed for non-SCLC cell lines *in vitro*.

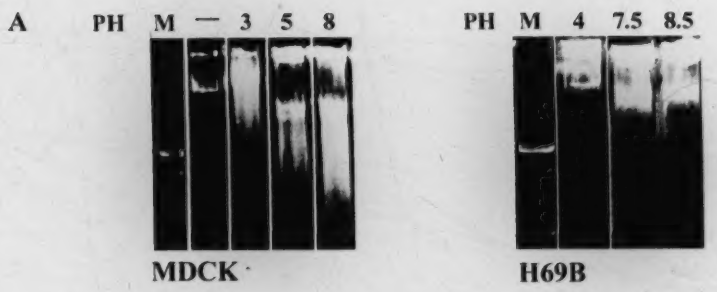
5.2.2 Evaluation of anchorage-independent growth

The ability of the SCLC cell lines, H69C and the derived adherent H69B cells, to form colonies in soft agarose, was examined to evaluate and compare anchorage-independent growth of these cells. In vitro colony-formation assays were carried out in complete medium containing 0.3% agarose (upper layer) and 0.6% agarose (lower layer) as described in the Materials and Methods. Representative morphologies of positive (Rat2-v-H-Ras) and negative (Rat2) colony formation of the controls, ras-activated transformed and nontransformed rat fibroblast cells, as seen by inverted microscopy, are shown in Fig. 21. Results of at least two independent experiments performed in triplicate indicated that H69C had limited growth in soft agarose after about 10-14 days whereas H69B cells did not grow at all in soft agarose.

Figure 21. Representative phase contrast micrographs of colony growth in soft agarose of v-Ha-Ras transformed rat2 fibroblast cells (A), non-transformed rat2 fibroblast cells (B) and H69C cells (C). Magnification, x 250 or at x 100 (inset).

5.2.3 Survival of H69B cells on polyHEMA-coated plates

Since the classical method for testing anchorage independence, i.e. colony formation in soft agar, has some drawbacks to it (346) we looked for an alternative *in vitro* method to determine if H69B cells have acquired a more "normal" response to an inappropriate environment and therefore a greater tendency to undergo apoptosis compared to the more tumorigenic H69C cells. We addressed the question of whether the H69B cells were more susceptible to apoptosis if they were released from their attachment to ECM by plating them on a polyhydroxyethylmethacrylate (polyHEMA)-coated plate that does not allow them to adhere to a substratum. Under these conditions, epithelial cells undergo apoptosis even in the presence of 10% (v/v) serum, while fibroblasts undergo apoptosis only under serum-free media conditions (205). H69B cells plated on polyHEMA in the presence of serum aggregate and were viable when tested in a MTT assay. Furthermore, when aggregrates of H69B cells grown on polyHEMA for 3-5 days were replated in tissue culture dishes, after initial attachment and spreading within a few hours, the replated H69B cells proliferated and formed a monolayer (Fig. 22B). This finding was confirmed by collecting H69B aggregates and floating cells from cells grown on polyHEMA-coated plates and assessing whether or not they undergo apoptosis using a DNA fragmentation assay (Fig. 22A). A regular 'ladder-type' pattern of DNA fragmentation due to endonuclease-driven digestion of DNA into mono- or oligomers of about 200bp is commonly considered a hallmark of apoptosis. During the apoptotic process, these DNA fragments are released from the nucleus to the cytoplasm where they remain stable for several hours. With cell lysis and extraction of



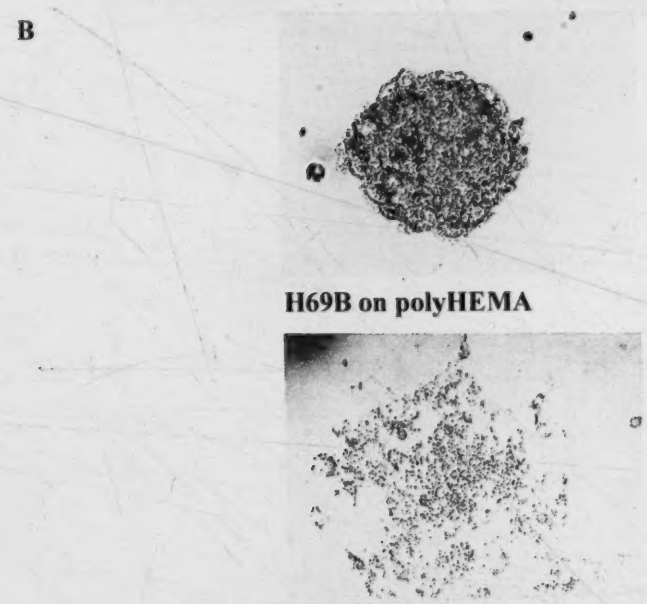


Figure 22. Effect of disruption of cell-matrix interactions on H69B cell survival. (A) effects on internucleosomal DNA degradation. MDCK epithelial cells (*left panel*) or H69B (*right panel*) were plated onto tissue culture dishes (-) or cultured on dishes coated with PolyHEMA (PH) for the time points indicated; cells were harvested and genomic DNA isolated and analyzed as described in Materials and Methods. (M is the kilobase marker). (B) Representative phase contrast micrographs of H69B cell morphology when cultured on polyHEMA (*top panel*) or transferred back to tissue culture plastic after culturing on polyHEMA for 5 days in complete medium (*bottom panel*). Magnification, x 250.

oligomeric DNA followed by DNA gel electrophoresis, fragmented DNA that has incorporated ethidium bromide can be visualized under UV light and captured digitally. Observation of the 'laddering' effect of DNA fragmentation is confirmation of apoptosis. As there is a delay in time with respect to nuclear fragmentation and appearance of fragments in the cytosol, sampling at various time points optimizes the detection of oligomeric DNA. In order to obtain a positive control for apoptosis as detected by the DNA fragmentation assay, H69B cells from monolayers were treated with the protein kinase inhibitor staurosporosine, which at high concentrations induces apoptosis. MDCK canine epithelial cells were also included as a positive control for cells that undergo apoptosis when deprived of substrate attachment (205). As Fig.25C shows, H69B cells undergo extensive DNA laddering when treated with staurosporine which is especially noticeable by 7.5 hr. Cells collected from polyHEMA-coated plates, however, did not show any signs of apoptosis by 8.5 hr even though the control MDCK cells started showing signs of DNA laddering by 3 hr of plating on polyHEMA (Fig. 22A). In addition to visual confirmation of staurosporine-induced death using inverted phase microscopy, analysis of H69B cells stained with Hoechst showed that only staurosporine-treated cells showed signs of condensed and fragmented nuclei typical of apoptosis (Fig. 23).

5.2.4 H69C and H69B cells exhibit differences in resistance to staurosporine-induced apoptosis

Given the high levels of Bcl-2 expression in the parental H69C cells compared to the derived H69B cells (Fig. 24), we wanted to determine whether there were any differences in resistance to apoptosis between the two cell lines. The protein kinase inhibitor, staurosporine, is a potent inhibitor of multiple protein kinases including PKC,

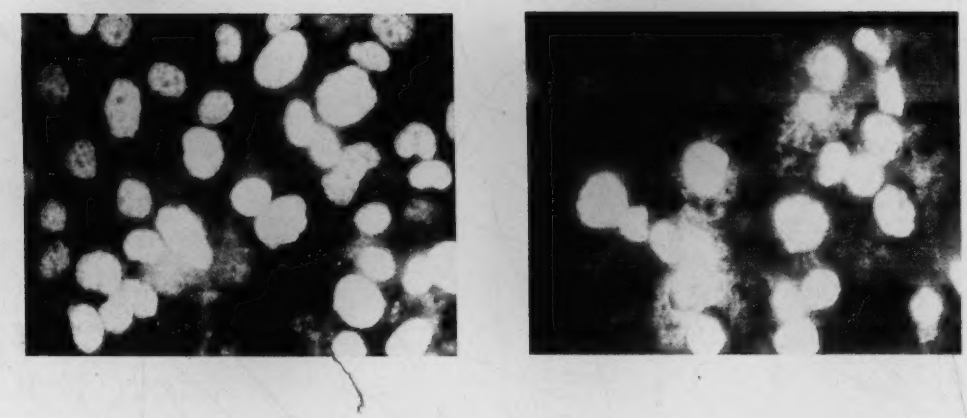


Figure 23. Induction of apoptosis in H69B cells by staurosporine treatment. Nuclear staining with Hoechst of untreated H69B cells (*left panel*) or cells treated with 1 mM staurosporine for 5 hours (*right panel*). Magnification, x100.

Figure 24. Expression of bcl-2 in H69B (*lane 1*), H69C (*lane 2*) and various control cell lines (*lanes 3-7*). Total RNA (15 μg) from different cell lines was processed for northern blot with bcl-2 probe. Arrows indicate the position of the 28S and 18S rRNA.

cAMP-dependent protein kinase, and receptor tyrosine kinases (347). Protein kinases are important in cellular signal transduction and in the induction of proliferation, differentiation, gene expression, and promotion of tumors. Inhibition of these kinases by treatment with staurosporine at high concentrations is established as a method to induce cell apoptosis. Analysis of staurosporine-treated cell cultures at various time points using the DNA fragmentation assay, showed that H69C cells are more resistant to staurosporine-induced cell death than H69B cells and control MDCK cells (Fig. 25). As shown in Fig.25A, untreated MDCK cells did not show any laddering by 10hr whereas staurosporine-treated MDCK cells show a DNA laddering pattern by 3 hr. H69B cells treated with staurosporine also showed DNA laddering within 4 hr (Fig. 25B) whereas H69C cells showed minimal amounts of apoptotic death by 4 hr and required 24 hr before there was a complete laddering effect, which had been observed in H69B cells as early as 7.5 hr (Fig. 25C).

5.2.5 Survival of H69B cells on ECM

Next, we sought to determine the growth characteristics of H69C and H69B cells on specific ECM substrates. Preliminary experiments under serum-free conditions examined the survival of H69B cells cultured on fibronectin, laminin, collagen I and collagen IV. ECM-coated 6-well tissue culture plates were examined visually under inverted microscopy and fields representative of the cell morphology across the whole plate were photographed (Fig. 26). The morphology of H69B cells grown on various

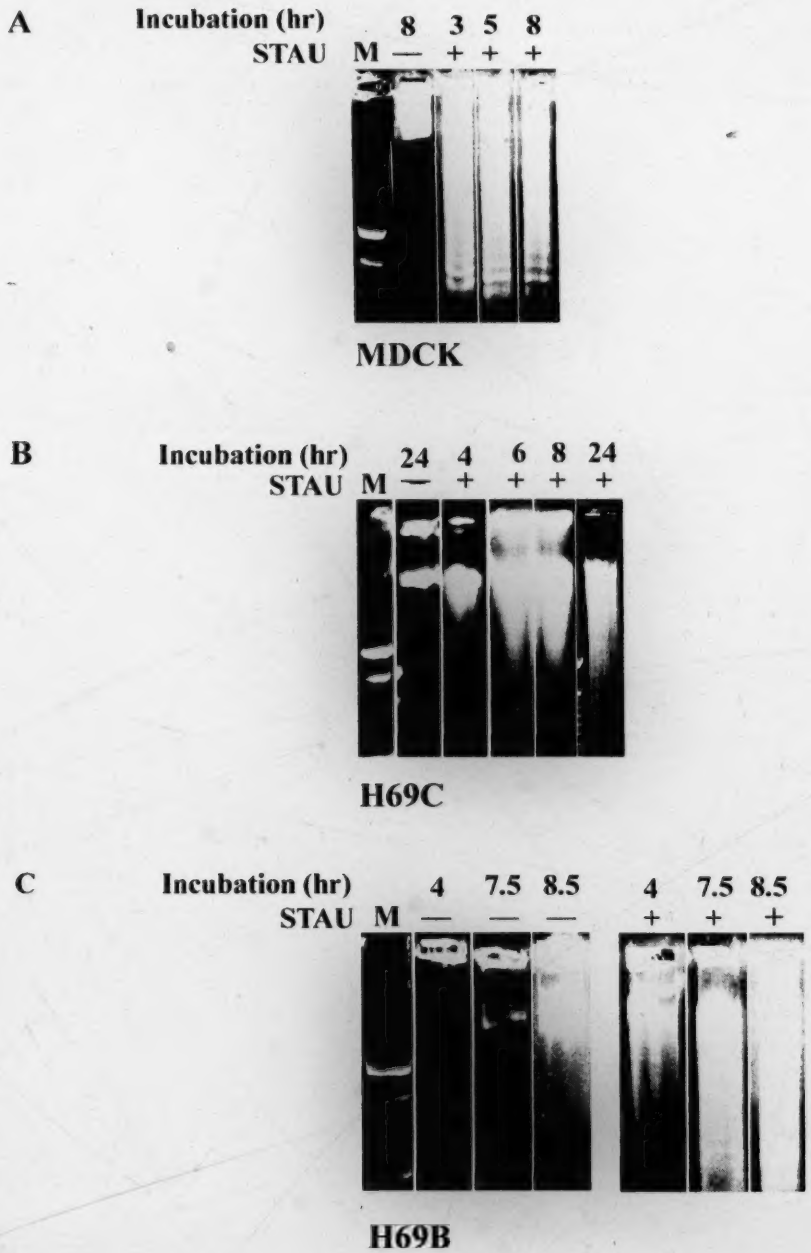


Figure 25. Differences in resistance to staurosporine (STAU)-induced apoptosis between H69C and H69B cells. Duplicate cultures of MDCK (A), H69C (B) or H69B (C) cells , incubated with (+) or without (-) 1mM staurosporine, were harvested at the time points indicated and genomic DNA isolated and analyzed as described in Materials and Methods (M is the 1 kilobase DNA ladder marker). Positive control, MDCK epithelial cells, show extensive oligosomal DNA fragmentation as evidenced by DNA laddering 3 hr after staurosporine treatment; H69B cells show complete DNA fragmentation by 7.5 hr, whereas H69C cells do not show complete DNA laddering effects until 24 hr after staurosporine treatment.

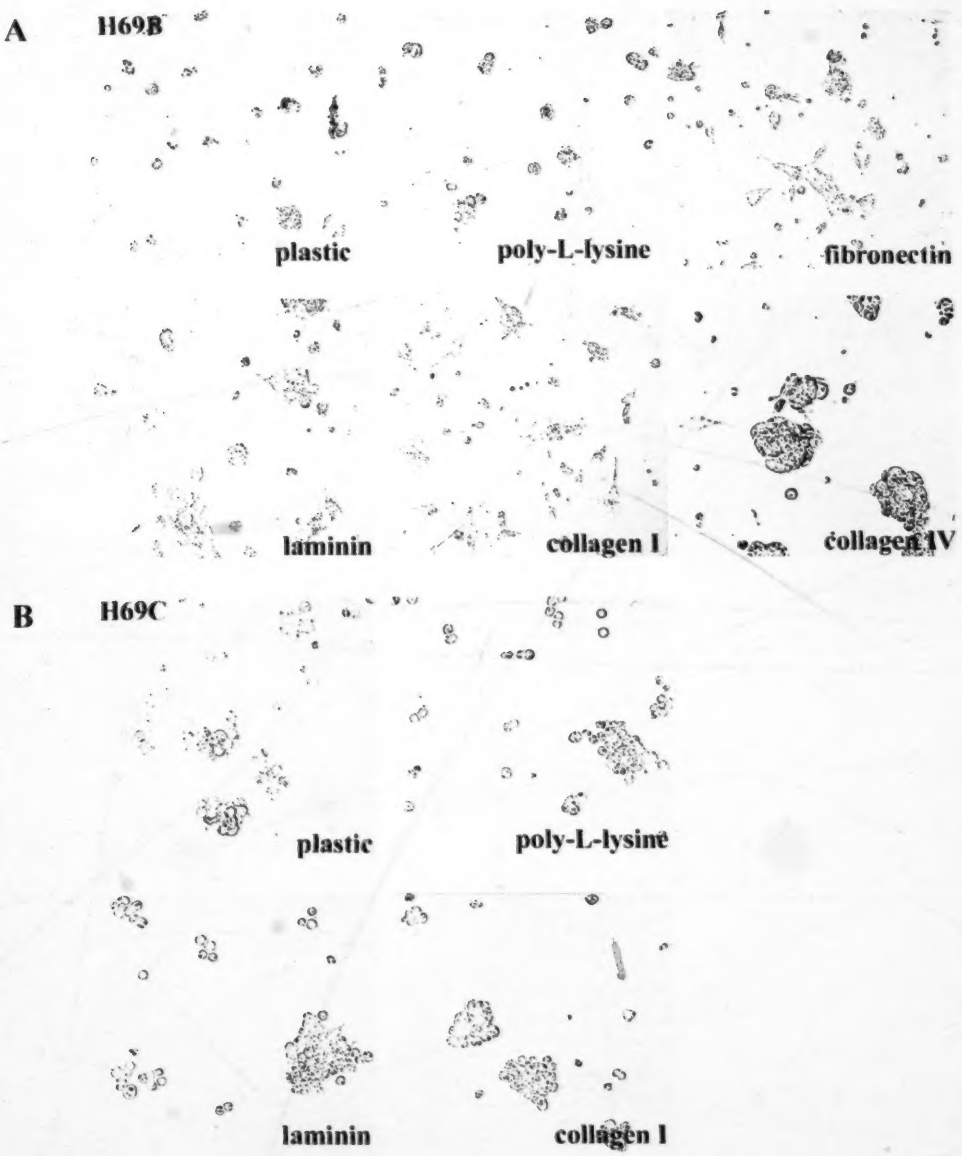


Figure 26. Survival of H69B (A) and H69C (B) cells grown on various ECM substrates under serum free conditions for 36 hours. Exponentially growing cultures of H69B or H69C cells were harvested and cultured at a density of 1 x 10^5 cells/ well in 6-well tissue culture plates coated with various ECM substrates as described in Materials and Methods. Phase contrast micrographs show representative morphologies of H69B and H69C cells. Magnification, 250x.

substrates is shown in Fig.26A. As a positive control for specificity of substrate and to assess levels of nonspecific adhesion, wells were coated with poly-L-lysine that promotes nonspecific attachment through ionic interactions. As a negative control, cells were also plated in the absence of substrate, on tissue culture plastic under serum-free conditions in which cells cannot produce ECM from soluble substrates present in serum. Whereas the cells plated on plastic or poly-L-lysine exhibited evidence of apoptosis by 36h as judged by cell detachment, membrane blebbing and condensed nuclei, the flattened epithelial morphology of spread H69B cells on ECM substrate was maintained much longer for all the substrates tested. However, there did appear to be some differences in the ability of the ECM proteins to promote cell survival: cells plated on collagen I appeared to be more resistant to apoptosis as judged by membrane blebbing compared to cells on fibronectin and laminin. Interestingly, results indicate that on collagen IV, H69B cells appeared to form cell clusters, however, their survival on this substrate was poor. When H69C cells were grown on these various ECM substrates under serum-free conditions, they did not seem to exhibit obvious differences in either morphology (Fig.26B). They did seem to spread more on laminin and survival was enhanced on ECM substrate compared to that of plastic and poly-L-lysine.

5.3 Discussion

The state of the cellular microenvironment in the context of growth factors and matrix components is coordinately monitored through the convergence of multiple inputs from cell surface adhesion and growth factor receptors. In particular, integrin signal transducers are sensitive to bidirectional feedback and maintain signals that balance the cellular decisions between pathways governing cell behavior. Significantly, different cell types such as fibroblasts and epithelial cells have evolved different mechanisms to undergo anoikis. Understanding how tumor cells resist the balance inherent in these regulatory mechanisms may lead to the restoration of normal function in transformed cells. In our SCLC experimental system, *in vivo* injection into immunodeficient mice showed reduced tumorigenicity in association with transdifferentiation to a more epithelial phenotype (H69B cells). In order to characterize further the functional significance of upregulation of adhesive complexes associated with the transition to an epitheloid phenotype, H69C and H69B cells were examined with respect to their growth rate, colony-forming ability, adhesion-dependent growth and susceptibility to induction of apoptosis.

In keeping with their transition to a differentiated phenotype, H69B cells have a decreased cell growth rate, doubling every 3-5 days, in comparison to that of the parental H69C cells. Growth rates similar to H69B have been observed for NSCLC cell lines in culture, consistent with the inherent plasticity of this SCLC cell line along a differentiation continuum (333). As H69B cells had a more extensive profile of integrin expression and exhibited increased adhesion to specific ECM substrates, we sought to

examine whether they would undergo apoptosis when deprived of an ability to attach. Consequently, we utilized two methods, the soft agarose assay, and culturing of cells on polyHEMA-coated plates and assessed cell growth and survival. The soft agarose colony formation assay measures anchorage-independent cell growth of transformed cells in culture. The ability to proliferate without firm attachment (anchorage-independence) is considered an indicator of *in vitro* tumorigenicity. In the present study, we found that while transformed fibroblasts rapidly formed massive colonies, H69C cells formed only a limited number of colonies in soft agarose while H69B cells did not grow at all under these conditions (Fig. 21). A similar study carried out by Khan et al. in which they tested both H69 SCLC cells and various derived adherent subpopulations found that only a fraction of the parental cells were clonogenic in soft agar (about 7%). Paradoxically, they found that their derived cells were more clonogenic in soft agar than the parental cells, despite being less invasive and non-metastatic *in vivo* (308). An alternative method of evaluating anchorage-dependent growth is to plate cells on a polyHEMA substrate. This prohibits cell attachment by blocking deposition of ECM proteins and has been used extensively to derive suspension cultures. In comparison to primary untransformed bronchial epithelial cells, which undergo apoptosis when cultured under conditions of prohibited cell-ECM adhesion, transformed cells are anchorage-independent and have lost substrate-dependent growth (232). When suspension cultures of H69B cells were cultured under conditions in which integrin-mediated cell-ECM adhesion was prevented, cell-cell aggregation occurred (Fig. 22B). Individual H69B cells on polyHEMA-coated dishes appeared apoptotic, whereas aggregates of H69B cells were found to be viable when analysed using an MTT assay although they did not proliferate. Furthermore, when

cell aggegrates cultured on polyHEMA for several days were transferred to tissue culture plastic they remained viable and regained the capacity of re-attaching and proliferating (Fig. 22B). Additionally, DNA extracted from H69B cells cultured on polyHEMA-coated dishes did not reveal the laddering pattern of fragmented DNA that is characteristic of apoptosis when analyzed on agarose gel electrophoresis (Fig. 22A). This was in contrast to the epithelial cell control MDCK cells which underwent apoptosis and showed extensive DNA laddering as early as 3-5 h of plating on polyHEMA. Thus, it appears that the formation of aggregates and presumed upregulation of cell-cell interactions by the H69B cells may contribute an anti-apoptotic signal that enables them to survive despite not being able to spread and adhere to substratum. It is possible that soluble factors secreted by these cells or present in the serum may contribute to the lack of expected apoptosis. A study by Valentinis et al. found that the tendency of their p6 fibroblast cells to aggregate when plated on polyHEMA in the presence of serum or IGF-I growth factor, correlated with that of cell survival although the mechanism leading to this aggregation was unknown. IGF-I was found to protect these cells from anoikis via FAK phosphorylation (346). A similar study using high-density (aggregated) bronchial epithelial cells plated on polyHEMA also looked at the possible contribution of soluble factors but did not find any differences in apoptosis with the addition of conditioned medium (232). However, they did find that aggregation was inhibited by culturing cells on polyHEMA-coated dishes in the presence of anti-α_V-integrin antibody or EDTA. Whether the apparent difference between these two studies reflects fundamental differences between mesenchymal and epithelial survival mechanisms or whether they are simply due to cell-type and context-specific differences is yet to be resolved. It would

be interesting to see if incubation of H69B cells cultured on polyHEMA-coated wells containing various anti-integrin, tetraspanin or cadherin monoclonal antibodies or RGD peptides (see section 1.3.1) would inhibit the aggregation or modify the apoptotic response. Several studies have shown a Ca^{2+}-dependent relationship between intercellular contact and inhibition of apoptosis such as that mediated by cadherins or integrins. In the current study, the mechanism of induced cell-cell interaction in H69B cells deprived of substratum adhesion is presently unknown. Whether this interaction is dependent on cadherins, integrins, or both, possibly involving tetraspanins, or whether there is a reversion to upregulation of N-CAM expression and consequent cell-cell adhesion of the type exhibited by H69C cells remains to be investigated.

Integrins, such as $\alpha_2\beta_1$ and $\alpha_3\beta_1$, mediate homotypic or heterotypic cell-cell adhesion, in addition to cell adhesion to matrix proteins. The ligand-binding mechanism of integrin cell-cell adhesion in epithelial cells is still largely undetermined. Direct integrin interactions such as $\alpha_2\beta_1$- $\alpha_3\beta_1$ and $\alpha_3\beta_1$- $\alpha_3\beta_1$ on opposing cells have been suggested to be important in cell-cell adhesion of keratinocytes (348). Transfection of integrin α_2 or α_3 into three different cell lines, however, did not enhance integrin-mediated intercellular adhesion making the existence of α_2/α_3 heterophilic or α_3 homophilic interactions controversial (349). Thus, sufficient evidence for the functional role of integrins in cell-cell interaction remains to be clarified and any additional role in the prevention of apoptosis needs to be examined. In one study involving lung bronchial epithelial cells, anti-integrin antibodies that blocked cell aggregation were shown to trigger apoptosis suggesting a role for integrin-mediated signal transduction in cell-cell adhesion and regulation of cell survival (350). Another study showed a correlation

between formation of cell aggregates and survival in epithelial-like A431 adenocarcinoma cells that was associated with upregulation of $\alpha_2\beta_1$ integrin expression (350). They showed that this $\alpha_2\beta_1$–mediated cell-cell aggregation protected A431 cells from cell death induced by the addition of high concentrations of EGF. Although signaling through FAK was implicated in this study as well as in other studies, the role of FAK and the context of its activation have been controversial and remains unclear (232).

Integrin-dependent EGF receptor activation has been shown to be important for anchorage-dependent cell survival. Integrins are found to co-cluster with growth factor receptors and direct co-precipitation studies have now shown them to be physically as well as functionally associated. Recent results by Yu et al. indicate that integrin $\alpha_2\beta_1$ not only physically associates with EGFR but also functions in serum-independent EGFR activation at cell-cell contact sites. They found that cell-cell contact is not necessarily required for association of EGFR with $\alpha_2\beta_1$ but is required for EGFR phosphorylation in serum-depleted conditions (351). They also found a requirement for cell-substratum adhesion. With the known juxtacrine activity of the membrane-anchored form of HB-EGF (pro-HB-EGF) and phosphorylation of EGFR by adjacent cells, soluble EGF ligands may not be required for EGF activation although the biological role for this is still not clear. EGFR activation at cell-cell contact sites may serve as a survival signal while excess stimulation of unassociated EGFR results in apoptosis. These apparent contradictions may perhaps be explained in that activation of the EGFR by high concentrations of EGF ligand without coordinate integrin ligation results in the triggering of apoptosis. Alternately, integrin ligation and activation of EGFR in the absence of soluble EGF may confer a survival signal, while a combined integrin and EGF

stimulation of the EGFR may result in the proliferation threshold being reached for cell cycle entry and progression.

Although direct co-precipitation of integrins and growth factor receptors has been observed, integrin interactions with growth factor receptors may be linked via integrin-associated proteins, perhaps in the context of lipid rafts (352). Integrins are involved in cis interactions with other cell surface proteins such as tetraspanins, CD47, GPI-linked receptors and EMMPRIN (123). Homotypic aggregation has been shown for CD9 (283). In particular, CD9-$\alpha_3\beta_1$ complexes are found localized at cell-cell contact sites. It has been suggested that the localization of these complexes to cell-cell contact areas in the absence of cadherins may indicate that they act at a step in cell-cell adhesion or recognition prior to cadherin adhesion (278). The authors also propose that the tetraspanin web may represent a ligand-independent mode of clustering integrin heterodimers. In fact, various combinations of growth factor-dependent or independent interactions between integrins, ligands and tetraspanins may lead to integrin-specific signaling complexes resulting in diverse signals such as polarization, intercellular adhesion or morphogenesis. Interestingly, CD9-$\alpha_3\beta_1$ complexes are found in association with the transmembrane precursor form of HB-EGF (proHB-EGF) or DTR, described in section 1.3.5.2. Specifically, DRAP-27 the monkey homolog of CD9 enhances the mitogenic activity of DTR/proHB-EGF, which functions as a juxtacrine growth factor (270). Juxtacrine activity is a term used to describe the interactions of an immobilized growth factor with neighboring cells (269). DRAP-27/CD9 modulates the juxtacrine activities of proHB-EGF, increases juxtacrine growth factor sensitivity 25-fold (280) and DT sensitivity by approximately 20-fold (353). It appears to stabilize DTR/proHB-EGF

on the cell surface by preventing processing and/or internalization. Since DTR/ proHB-EGF alone may not be stably retained on the cell surface, association of DRAP-27/ CD9 with DTR/ proHB-EGF may stabilize DTR/ proHB-EGF thereby upregulating DT binding and mitogenic activity (175). It has been reported that proHB-EGF protects against apoptosis whereas the soluble form did not (354). Perhaps the functional role of tetraspanins in connection with proHB-EGF regulation may explain not only its role in cell proliferation (mentioned in section 4.3.4) but also in regulating cell survival or death. For example, in CHO-transfected cells CD9 and CD82 were shown to have an apoptotic-inducing effect which was, interestingly, glycosylation-dependent (306). EGF and EGF-R are known to be increased in SCLC/ NSCLC and contribute to autocrine/paracrine mechanisms (Fig. 1, section 1.1.3). Whether EGF is a factor in the current study is still unknown.

Cross-talk between cell-cell adherens junctions and formation of focal adhesions and stress fibers following EGF stimulation of serum-starved endothelial cells has recently been described (355). Several lines of evidence suggest that there is cross-talk between cadherin-, integrin and CAM-mediated adhesion systems although as yet much of this area remains undefined. In keratinocytes, studies have shown that cadherins downregulate integrin expression during keratinocyte differentiation. One report demonstrated a five-fold increase in N-cadherin expression by ectopic expression of α_5 or $\beta 1$ integrin expression in myoblasts which led to cessation of motile activity through enhanced cell-cell aggregation (356). In another study, prevention of neural crest cell migration using RGD peptides or antibodies to fibronectin and to β_1 and β_3 integrins caused rapid N-cadherin-mediated cell clustering (357). In mouse mammary epithelial

cells, plating on function-blocking anti-β_1 integrin antibodies, or on specific matrix components, resulted in significant upregulation of P-cadherin expression (358). In our system, it is possible that the presence of N-cadherin in H69B cells, concomitant with increased cell-contacts and cell-density, may be able to mediate survival of cells without substrate adherence. Interestingly, treatment of H69 SCLC cells with C3 exoenzyme, which acts to constitutively activate Rho small GTPase, was found to induce increased aggregation. However, this was suggested not to be through a cadherin-mediated mechanism (229). Intriguingly, a recent report implicates a role for N-CAM in progression of tumor metastasis through stimulation of β_1 integrin-mediated cell-matrix adhesion by activating FGFR signaling (359). Importantly, cadherins, integrins and CAMs serve both structural adhesive functions in anchoring the cell to the actin cytoskeleton as well as acting as signaling receptors that affect cell behavior. With shared regulatory cytoskeletal molecules such as actin, α-actinin and vinculin found at both cell-cell junctions and cell-matrix adhesion sites, it is not at all suprising that contact-mediated inhibition of proliferation and cell migration is coordinated by cross-talk between these adhesion systems (360). A key player in both cell-cell adherens and cell-matrix interactions, ILK is a likely candidate for converging integrin/cadherin-mediated signaling pathways regulating cell proliferation and survival mechanisms. Recent work shows that ILK binds directly to β_1 and β_3 integrin cytoplasmic domains and can induce EMT in two distinct epithelial cell types (78). This occurs concomitant with downregulation of E-cad expression via β-cat translocation and transcriptional activation of β-cat/LEF1-gene targets in the nucleus (361).

5.3.1 Importance of the epithelial gene expression program

Genes that initiate EMT/MET also often confer resistance to various forms of apoptosis, including anoikis. These genes include various signaling molecules such as *raf-1*, transcription factors (snail/slug family repressors), and cell-cell adhesion molecules (362). The relationship between phenotypic conversion and sensitivity to anoikis needs to be investigated. Recently, *bcl-2* transcription and protection against anoikis has been linked to specificity of integrin ligation in CHO cells transfected with the $\alpha_5\beta_1$ integrin fibronectin receptor. The authors show that the *bcl-2* promoter is regulated by FAK, Shc and PKB/Akt signaling molecules (363). The importance of cell death pathways in carcinogenesis and treatment-resistance has led to several novel strategies targeting apoptotic mediators, such as Bcl-2, that are frequently dysregulated in SCLC (e.g., bcl-2 antisense). Given the differences in Bcl-2 expression in H69C and H69B cells, it would be interesting to see if Bcl-2 expression were increased in the aggregated H69B cells, as one study showed that prevention of apoptosis by cell-cell adhesion/aggregation was associated with Bcl-2 expression (232). Bcl-2 may represent a mechanism by which both cell-matrix and cell-cell interactions through integrins can prevent apoptosis. Activated Ras can prevent downregulation of Bcl-X(L) triggered by detachment from the extracellular matrix (364). Ras is known to play a role in protecting cells from anoikis through activation of multiple pathways although the relative importance of these pathways depends significantly on the cell context in signaling pathways that control anoikis. Ras has a pivotal position, downstream of both growth factor and cell adhesion receptor signaling pathways. Thus, aberrant Ras activation provides a selective advantage for cancer cells allowing them an increased survival time in the absence of matrix

attachment in which to facilitate eventual reattachment and colonization of secondary sites. Overexpression of both *ras* and *myc* genes affect endocrine and epithelial features of SCLC (14). Overexpression of *myc* has been correlated to patient prognosis as well as to morphology and biology of SCLC cell lines. Downregulation of the *myc* oncogene by differentiation signals or by antisense expression has been associated with decreased proliferation, clonogenicity in soft agar and induction of apoptosis (365). A relationship between *c-myc* overexpression and decreased $\alpha_3\beta_1$ integrin expression has been found in SCLC cells (332). Other studies have found an inverse relationship between N-myc and β_1 integrins in neuroblastoma cells (366). Interestingly, expression of *c-myc* which is highly expressed in H69C cells, is down-regulated in H69B cells (Dixon, unpublished results). The finding in the current study, that adherence to plastic, or non-specific adherence to poly-L-Lysine-coated dishes did not prevent apoptosis of H69B cells at 36 hours supports the concept that specific receptor interactions mediated by integrins are important in regulation of H69B cell survival. Preliminary experiments did suggest that there appear to be some differences between H69C and H69B cells in the ability of various ECM proteins to promote cell survival under serum-free conditions. Further experiments are required to examine how many of these cells are apoptotic and to determine the degree of survival under conditions of complete medium or added growth factors. A switch of gene expression in SCLC cell lines, reverting them to a more differentiated, non-tumorigenic phenotype may represent the basis to develop new strategies for the therapy of this aggressive tumor.

CHAPTER 6 CONCLUSION

6.1 Summary

Tumor progression in the lung is now believed to result from a combination of genetic change superimposed by epigenetic effects that create phenotypes resistant to regulation by cellular control mechanisms. Divergent differentiation of lung tumor cells followed by environmental selection leads to the generation of clonal subpopulations that are highly adapted for aggressive growth and metastatic capability. In particular, unregulated, pathological EMT with cellular regression to embryonic-like phenotypes is relevant to the mechanisms governing the formation and dissemination of metastatic tumor cells (23). The process of EMT/MET conversions and integration of EMT signals at the membrane level is regulated by specific cell adhesion receptors. In this regard, adhesion complexes have been shown to be critically involved in tumorigenesis and the ability of malignant cells to metastasize (212). Since successful treatment of SCLC will depend on the development of multidimensional therapeutic approaches, the present study utilizes an experimental SCLC system to examine the relationship between differentiation status and changes in cell adhesion pathways as a pre-requisite for invasion and metastasis.

The results of this study show that transdifferentiation of a non-adherent parental SCLC cell line to a derivative adherent subpopulation, associated with reduced *in vivo* tumorigenicity, appeared to be due to phenotypic rather than genotypic differences. Expression of vimentin and cytokeratin intermediate filaments as markers of mesenchymal and epithelial phenotype, respectively were altered between H69C and

H69B cells. Concurrent with transition to an epithelioid morphology, H69B cells have upregulated expression of cadherin and catenin adhesion proteins which participate in structural and signaling complexes responsible for maintaining epithelial versus mesenchymal potential. Ultrastructural examination of the two cell types by electron microscopy revealed the formation of ultrastructurally recognizable adhesion complexes only in the adherent cells. Upregulation of these protein complexes was associated with the downregulation of mesenchymal N-CAM which is prominently expressed in the tumorigenic H69C cells. Increased substrate adhesion in H69B cells was found to be associated with differences in integrin repertoire, cell surface expression and in regulation of β_1 integrin heterodimer processing. In keeping with the increased cell surface localization of integrins in H69B cells, transdifferentiation was associated with an alteration in the ratio between precursor β_1 and mature glycosylated β_1 integrin. Differences in integrin expression and subcellular localization are paralleled with changes in tetraspanin synthesis, cell surface expression and increased integrin-tetraspanin complexes. These changes corresponded with enhanced integrin function in substrate-specific adhesion although an effect on regulated motility and invasive cell behavior has yet to be determined. In addition, the current study also shows that differences in differentiation state and tumorigenic potential are associated with altered growth rate, colony-forming ability and susceptibility to induction of apoptosis *in vitro* in the two cell lines.

6.1.1 Phenotypic plasticity of SCLC cells

The characterization experiments described here have generated many additional questions as to how normal and pathological adhesive mechanisms during lung cell differentiation may be regulated and suggested many interesting avenues for further study. The development of cellular heterogeneity that occurs during progression of lung cancer proceeds at different rates and to different degrees. Subtle changes resulting from differentiation may lead to complex cell populations and have profound implications for tumor behaviour. With the ability to manipulate differentiation status it may be possible to modulate response to treatment. During subcloning of H69B cells by limiting dilution (as described in the Materials and Methods), it was observed that there were various clonal phenotypes exhibited that were generated from single cells and were maintained in long-term culture. Representative morphologies of the main subtypes as seen by inverted microscopy are shown in Appendix I. The two main cell types were epithelioid and fibroblastic. However, even with the epithelioid clones there was a range in morphological appearance with respect to transparency and cellular boundaries. In one clone, for example, the cells looked distinctly dendritic but grew poorly. In contrast, the growth characteristics of the fibroblastic cell types were much more rapid than the epithelioid cells and were less adhesive (as measured by trypsin detachment). The ability of SCLC cells in culture to exhibit such differing phenotypes may reflect the clinical behavior of the original tumor cells. The known links between SCLC and non-SCLC tumors in terms of phenotypic characteristics may allow the exploration of potential transitions between the phenotypes and elucidate their role in the development of treatment resistance in patients.

6.1.2 Role of integrins and tetraspanin function in regulating adhesive and de-adhesive behavior in SCLC migration and invasion

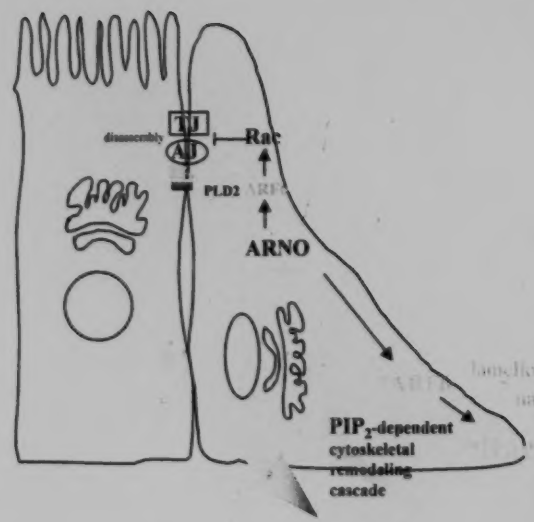
ECM differentiation signals are generated through integrin receptors. In one recent study, re-expression of $\alpha_2\beta_1$ in a breast cancer/ mammary cell system resulted in reversion and maintenance of a differentiated phenotype (367). This may explain the presence of this integrin at cell-cell contacts, perhaps in association with tetraspanins as engagement of tetraspanins has been observed to functionally induce morphogenesis in various cell types (172). Assembly of tetraspanin-specific integrin signaling complexes may be one means by which differential integrin signals are generated (273). Loss of integrins in tumor cells can render them unresponsive to positive or negative growth and differentiation signals from the ECM (368). In light of this, the lack of integrin expression in H69C cells, in particular, the deficiency in $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrins could allow the establishment of a clear causal relationship in this experimental system between integrin-specific expression and function with respect to phenotype, morphology, cell behavior and tumorigenicity. Transfection of cDNA's encoding α_2 and α_5 integrin subunits into H69C cells should allow direct testing of this hypothesis. Considering the overall lack of integrin expression in these cells, it would be interesting to determine the consequences of upregulated expression of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrin receptors and any similarities to the H69B cell phenotype, in terms of tumorigenicity, adhesion, migration or differentiation. As well, $\alpha_3\beta_1$ expression is known to influence morphology and anchorage-dependent growth *in vitro*, and an ability to form tumors *in vivo* (369). Perhaps the overexpression of wildtype and/or mutant $\alpha_3\beta_1$ in the H69C cells may allow further determination of whether altered function of $\alpha_3\beta_1$ and associated tetraspanins

directly contributes to the H69B phenotype. Now that we have established the lack of tetraspanin-integrin complexes in highly metastatic SCLC cells (H69C), and their presence in differentiated SCLC cells (H69B), it will be necessary to examine further the possible expression of other tetraspanins such as CD151, which is found in $\alpha_3\beta_1$ complexes at the adherens junctions. It will also be important to examine the potentially distinct roles of these different tetraspanin-integrin combinations in assays such as cell spreading, directed cell migration/invasion, and morphology in 3-dimensional matrigel cell cultures, comparing whether such individual functions are altered between H69C cells and H69B cells. To this end, initial experiments were performed to express CD9 and CD63 in H69C cells, which are almost CD9-deficient and which only weakly express CD63, to look for any possible effects on the morphology of these cells. Plasmid and vector DNA for CD9, CD63 wildtype, CD63 glycine mutation and CD63 phenylalanine mutation constructs were prepared and electroporated into H69C cells as described in Materials and Methods. Briefly, site directed mutagenesis of a human CD63 cytoplasmic tail sequence was used to substitute a glycine or phenylalanine residue for a critical tyrosine residue in a conserved Glycine-Tyrosine dipeptide motif found in lysosomal membrane glycoproteins such as CD63 (Appendix IIA) (208). The presence of this motif is thought to be an intercellular sorting signal as well as an internalization signal in endocytotic pathways. Thus, it was hoped that a mutation in this CD63 motif would determine 1) whether CD63 sorting is dependent on a tyrosine-containing cytoplasmic tail and therefore if this motif is necessary and sufficient for transport of CD63 from the Golgi compartment to lysosomes or in intracellular exocytic/endocytic pathways and 2) whether the mutation is sufficient to cause accumulation or redistribution of the mutant in

order to study the consequences of CD63 mislocalization on protein-protein associations and ultimately cell behavior. A preliminary Northern Blot (Appendix II) of cells selected in G418 indicates that mRNA expression of CD63 in the transfectants is increased compared to that of the parental untransfected H69C cells and the vector control. None of the CD9 constructs seemed to transfect H69C cells succesfully. It is possible that the low levels of endogenous CD9 expression in H69C are due to an inability of these cells to maintain increased expression and therefore exogenous expression of the construct is toxic or lacks appropriate transcription factors. Observations of CD63-transfected cells in culture could not detect any differences in adherence to tissue culture plastic, but the morphology of the CD63 phenylalanine mutant showed slight differences in cell size and aggregation compared to CD63 wildtype-expressing and parental cells. Further analysis using flow cytometry to examine cell surface expression of CD63, tetraspanins and integrins in the H69C cells transfected with mutant CD63 will provide information on subcellular localization and trafficking events which may be affected in these mutants. In addition, designing a construct with an epitope tag (e.g. 9kDa hemagglutinin) to discriminate between endogenous and exogenous CD63 would allow the design of more specific experiments to target CD63 trafficking function. Tetraspanins are believed to be involved in the localization of integrins to vesicle and membrane microdomains. Evidence linking tetraspanin function to biochemical signaling pathways within the cell is finally beginning to emerge (Fig. 27). Interestingly, following stimulation of the heterotrimeric G protein-coupled muscarinic acetylcholine receptor (mAChR), SCLC cells exhibit increased adhesion to collagen type IV through activation of PKC (370,371).

A

Figure 27. Proposed model of tetraspanin (TM4)-integrin interactions in the transition of a typical adherent epithelial cell to a mesenchymal-like motile phenotype. Integrins and the regulation of their supply at the cell surface depends on the biosynthetic pathway in a regulated endocytic /exocytic cycle in the following sequence: 1). Integrin activation by ligand or associated regulatory proteins in response to growth factor stimulation results in assembly of focal complexes some of which become internalized and recyled back to early endosomal or perinuclear recycling compartments where feedback loops modulate organization and target polarized integrin-specific delivery to complex-specific domains at the cell surface (326). **2).** Cytoplasmic proteins which are focal contact components such as talin may regulate integrin entry into the biosynthetic pathway. Phosphoinositides are thought to stabilize talin interactions with ligand-activated integrin. Internalization of activated integrin-ligand bound to talin may stimulate integrin exit from the ER or ER to Golgi intermediate compartment (ERGIC) by disclosing a membrane targeting sequence on the integrin for vesicle transport along the CSK, thus speeding up the rate of integrin through the biosynthetic pathway (252). It is possible that specific integrin association with TM4s determines whether or not it is recycled and therefore whether or not integrin-containing focal complexes mature into focal adhesions (155). **3).** Tetraspanins may facilitate recruitment of activating enzymes such as PI4-K and PKC and link them to integrin function (285,287). TM4 association with PI4-K may participate in the formation of the PI4,5 (PIP_2)and other signaling intermediates. PI lipid composition determines differential recruitment and activation of specific PKC isoforms as well as PKC substrates such as MARCKs. **4).** Integrin associations with specific TM4s may play a role in delivering and removing integrins from the cell surface, and their intracellular localization in vesicle trafficking events in conjunction with small GTPases (dynamin, Rab, Arf, Rac, Rho, Cdc42) which coordinate targeting, trafficking, membrane fusion and the dynamics of actin polymerization events. Exchange factors (GEFs) for small GTPases are recruited and regulated by their pleckstrin homology (PH) domains which bind to membrane phospholipids and are therefore responsive to localized alterations in membrane phospholipid composition (328). TM4-TM4 associations cluster integrins and other molecules into higher order complexes forming a multimolecular TM4 web. **5).** The glycosylation status of the cell determines organization of glycolipid-enriched membrane domains and contributes to integrin-TM4 interactions (275). TM4 may interact with lipids, such as GM3, via its palmitolyation state which would then contribute to the ability of TM4s to associate in various combinations or to oligomerize. This may determine the threshold for TM4 partitioning into lipid rafts (or detergent-resistant microdomains) (327). The degree of myristolylation or palmitoylation may affect which lipid rafts associate with different isoforms of PKC, MARCKs and other signaling molecules. Lipids determine the partitioning of integrins and other proteins into vesicles or lipid rafts within the cell **6).** The availability of divalent cations is also important. Cations appear to be important in TM4-TM4 interactions. **7).** The phosphorylation state of the integrin may play a role in modulating it's ability to recruit and bind cytoskeletal and signaling proteins which make up the dynamic nature of the molecular focal complex and thus the ability of the focal complex to disassemble,undergo turnover or to mature into more stabilized focal adhesions. **8).** In stabilized adhesion, the endocytic /exocytic cycle is decreased whereas in migration repeated transient increases in intracellular free Ca^{2+} activates enzymes such as Ca^{2+}-calmodulin activated protein phosphatase 2B/ calceineurin (CN) which leads to severing of cell-matrix contacts at the rear of the cell, possibly with internalization and recycling of integrin receptors and adhesion components back to the leading edge (144). Kinases such as PI3-K may be required for activation of dynamin-influenced endocytosis and therefore regulate the rate of endocytosis/recycling (156). PI3-K, in response to a growth factor stimulus , may act on PLC-γ in the membrane to enhance DAG levels which would then activate PKC. Activated PKC is then recruited to integrin complexes which form a complex with actin-binding regulatory proteins such as ERM proteins, which affect CSK turnover and maintenance of cell shape and lamellopodial extension.


Rac
PLD2
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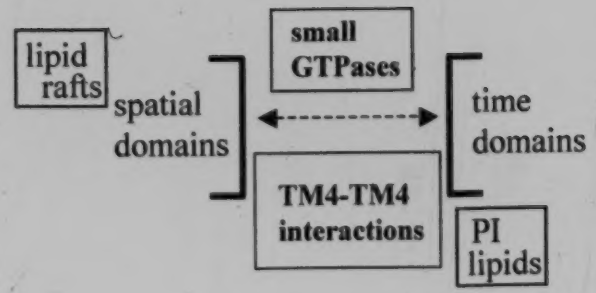
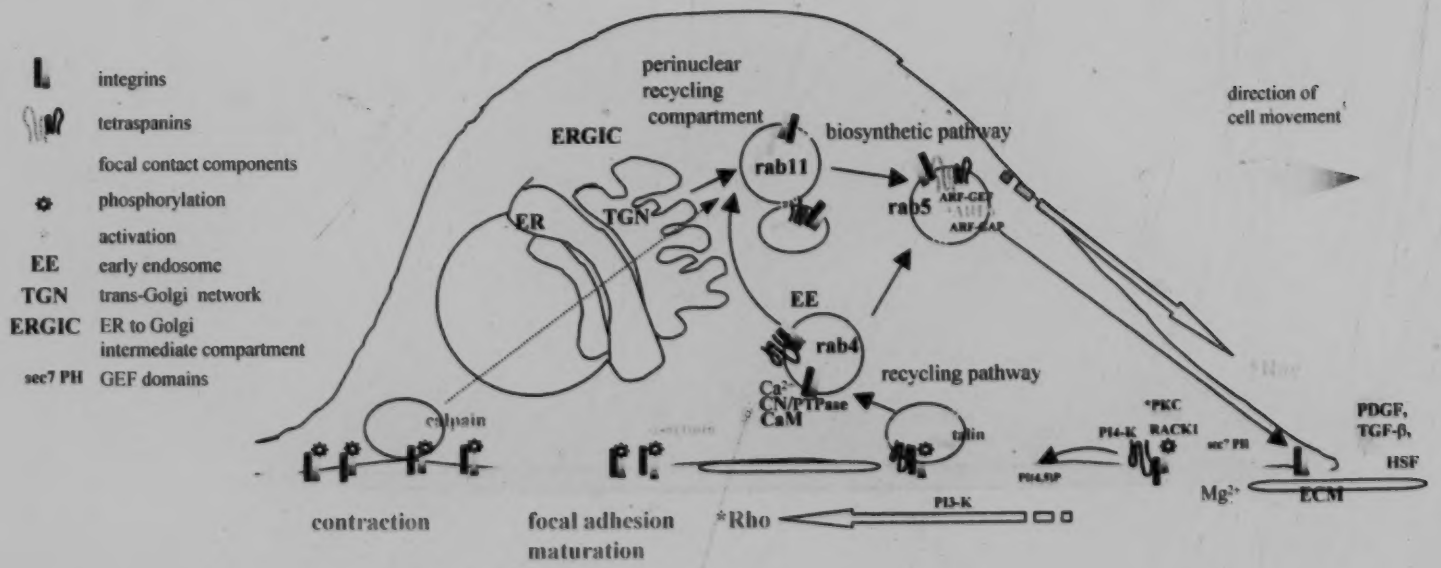
remodeling
cascade


lipid rafts
spatial domains
small GTPases
time domains
TM4-TM4 interactions
PI lipids


integrins
tetraspanins
focal contact components
phosphorylation
activation
EE early endosome
TGN trans-Golgi network
ERGIC ER to Golgi intermediate compartment
sec7 PH GEF domains
perinuclear recycling compartment
ERGIC
biosynthetic pathway
direction of cell movement
rab11
rab5
ER
TGN
EE
rab4
recycling pathway
Ca2+
CN/PTPase
CaM
calpain
talin
*PKC
PI4-K
RACK1
PI4,5P
PDGF, TGF-β, HSF
Mg2+
ECM
PI3-K
*Rho
contraction
focal adhesion maturation

recent study has shown that mAChR activation diminishes the ability of cells to detach from the substratum, resulting in diminished migration (372). The fact that stimulation of this receptor also promotes cadherin-mediated cell-cell adhesion and decreased SCLC proliferation may provide a useful approach to assigning specific mechanistic roles for cadherin, integrin and tetraspanin-related signaling pathways in regulating EMT. Better understanding of the mechanisms underlying the invasive and metastatic potential of SCLC may contribute to the design of therapeutic strategies to limit tumor invasion and metastasis.

6.2 Future Directions

Current studies by Bissell and colleagues have led to the suggestion that tissue architecture is the ultimate tumor suppressor (373). They propose that the 'dominant paradigm wherein multiple genetic lesions provide both the impetus for, and the Achilles heel of, cancer might be inadequate to understand cancer as a disease process' (374). Furthermore, there are several lines of evidence to illustrate the influence of the microenvironment in the evolution of the malignant phenotype (374). Utilizing a breast carcinoma epithelial cell model system which addresses the mechanisms involved in malignant conversion in the breast they have shown that treatment of tumorigenic breast cancer cells with β_1 integrin function-blocking antibodies resulted in phenotypic reversion of these cells in 3-dimensional gel cultures, to that of a non-tumorigenic acinar polarized phenotype (373). *In vivo* injection of these β_1-treated cells into nude mice resulted in a significantly reduced tumor number and size. Inhibition of tumor cell β_1 integrins was also able to rescue adherens junction assembly establishing a definite link between integrin signaling, adherens junctional assembly and the coordinated formation

of a differentiated tumor structure. Suprisingly, treatment of an earlier passage of non-tumorigenic breast epithelial cells with β_1 integrin function-blocking antibodies resulted in apoptosis *in vitro*. These results indicate that not only may dysregulation of integrins play a causal role in the expression of malignancy in human epithelial breast cells but that their cell-surface manipulation can restore tissue form and function and reduce tumorigenicity *in vivo*. They postulate that, in their system, the re-balancing of signals transduced by β_1 and β_4 integrin receptors allowed the breast cacrinoma cells to sense their microenvironment appropriately and thus re-establish appropriate tissue organization and function. Thus, even in the presence of prominent mutations, amplifications and deletions, signaling events linked to the maintenance of normal tissue architecture are sufficient in this case to restrain tumorigenic behavior of cells. Therefore, changes in tissue structure are critical for expression of the malignant phenotype and ECM receptors are important modulators of this effect. This evidence may explain the poorly understood phenomenon of spontaneous tumor reversion and tumor cell dormancy. Thus, cellular and tissue architecture may act as the most dominant tumor suppressor of all and in this way, the phenotype can—and does—override the genotype when normal tissue architecture is maintained (373). A growing body of evidence suggests that cells have evolved the capacity to trigger development via common pathways (136). That is, a variety of environmental cues can be translated into a few possible cell fates or distinct phenotypes such as differentiation and quiescence, proliferation or apoptosis. An example of this is the ability of tumor cells to exhibit vascular mimicry, in which they can recreate their own vascular system without the requirement of angiogenesis (that is, blood cells or the blood system) (375). This also

highlights the enormous potential of both normal and malignant cells to reorganize their genomes to create new forms and tissues if appropriate microenviromental stimuli are present. Current studies are addressing how regulation of tissue-specific gene expression in differentiated cells is maintained, in part, through control of chromatin structure and histone acetylation by information from the microenvironment (376). Also being investigated, are the functional reversions mediated by normalization of ECM signaling that alter patterns of histone acetylation and which can return the chromatin structure to that of a normal, differentiated cell. The emerging picture is that EMT involves the integration of new signals originating outside the cell and generating a conversion in the transcriptional control of the epithelial phenotype through activation of several distinct families of transcription factors (79,80,377). As Dr. Bissell and colleagues propose, "there is now ample evidence to suggest that terminal differentiation may indeed be a perception rather than a reality, a possibility raised almost two decades ago" (375).

Phenotypic plasticity in lung tumor progression is a clinical problem. In the experimental system developed here, highly invasive SCLC cells exhibit a pluripotent and deregulated phenotype. Treatment of these cells with a differentiating agent induced transformation to a more epithelial-like cell morphology which was associated with upregulated expression and activity of adhesion complexes and reduced tumorigenicity *in vivo*. The findings characterized in this experimental system will allow a more specific determination of the contribution of adhesive differences and their corresponding signaling pathways to the invasive status of lung carcinoma cells. Further investigations into the mechanisms of communication between the tissue architecture of the extracellular environment of the lung cell and its intracellular signaling pathways are

much needed. Understanding the pathways that define the phenotypic versatility of cell behavior will lead to an understanding of how uncontrolled proliferation, invasion and resistance to homeostatic mechanisms arise within the human body and thus eventually to the prevention and elimination of cancer.

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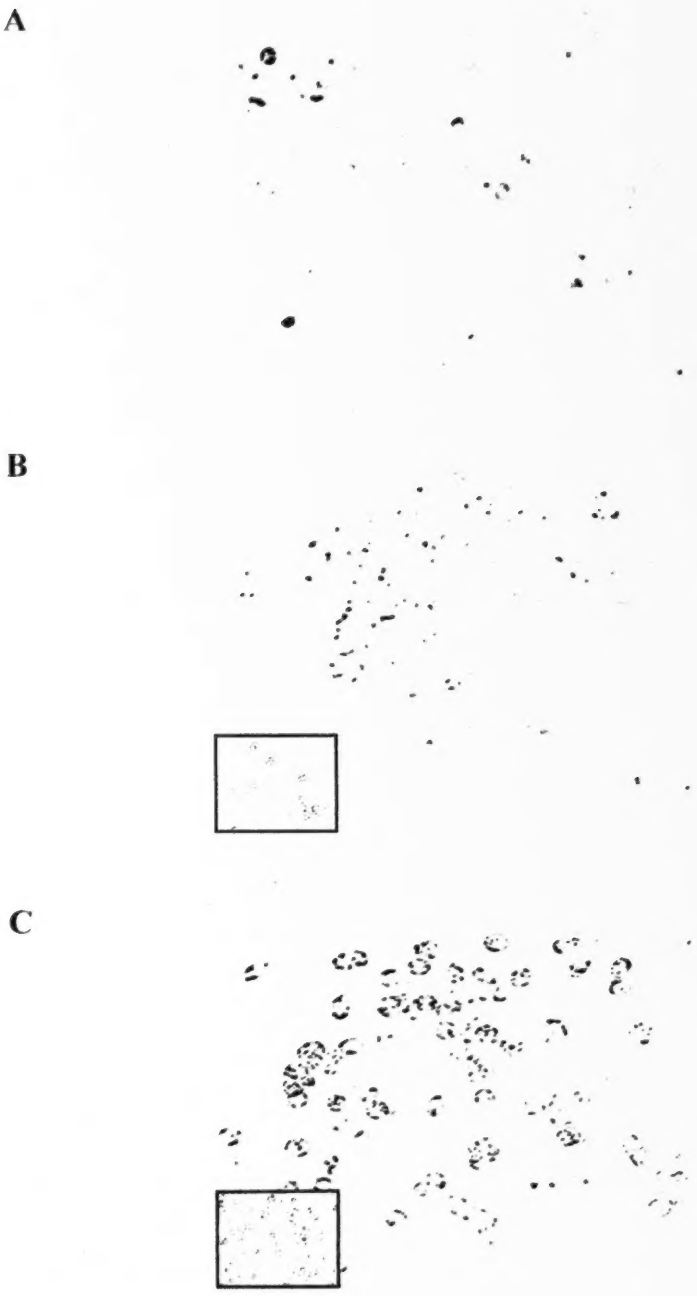
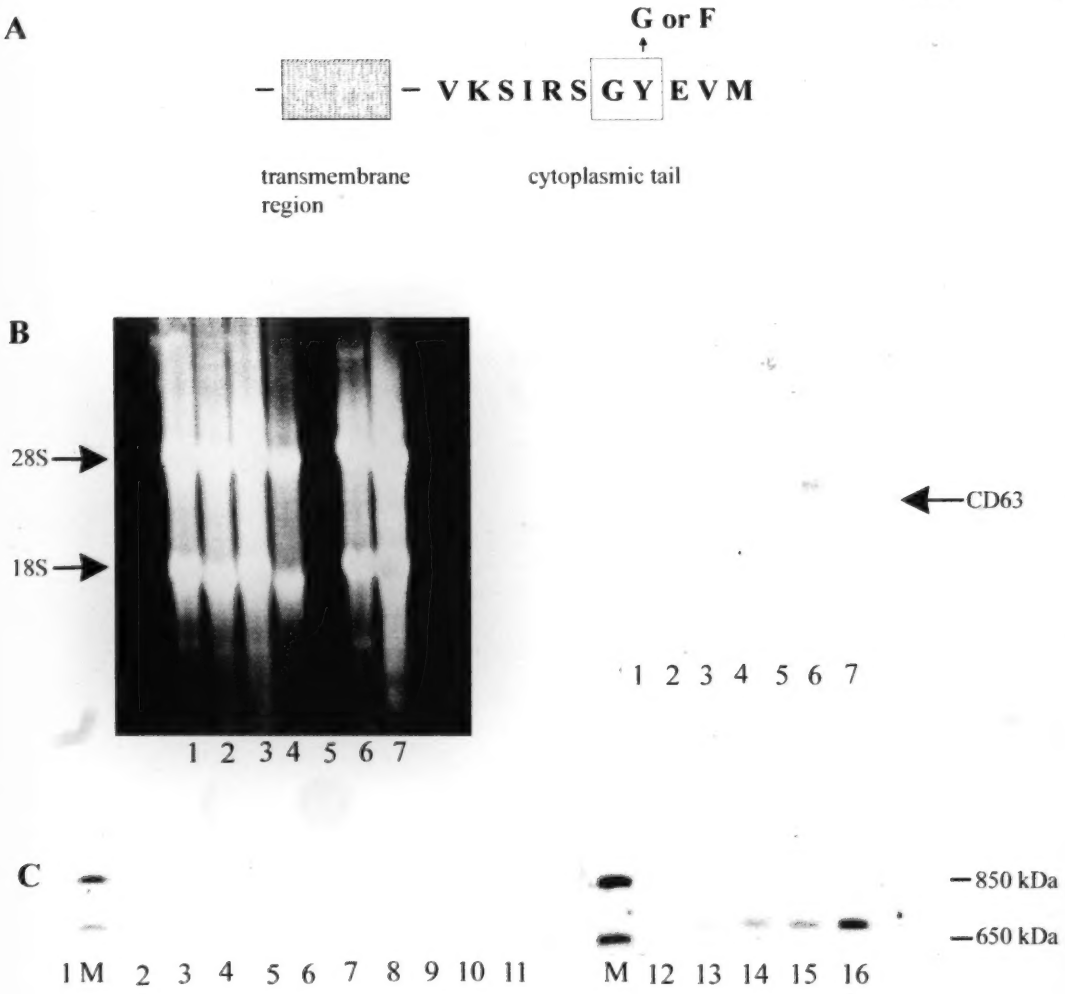


Appendix I. Phenotypic plasticity of H69 derived adherent subpopulations after subcloning by limiting dilution. Phase contrast micrographs show representative epithelioid (A, B) and fibroblastoid (C) cell morphologies. Magnification, 250x (insets in B,C are 100x).



Appendix II. Expression of CD63 in H69C and CD63-transfected H69C cells. (A) Schematic showing CD63 conserved Glycine-Tyrosine dipeptide (135). **(B)** RNA was isolated from exponentially growing cultures of H69C (*lane 1*), vector only pcDNA (*lane 2*), wildtype CD63-transfected cells (*lane 3*), CD63-glycine mutant (*lane 4*), no RNA (*lane 5*), CD63-phenylalanine mutant (*lane 6*) and CD63-negative A20 cells (*lane 7*), and separated by denaturing agarose gel electrophoresis (15 ug/lane) as described in Materials and Methods. Arrows indicate the position of the 28S and 18S rRNA (*left panel*). RNA was transferred to nylon membranes and hybridized to a ^{32}P-labeled probe for the CD63 tetraspanin gene (*right panel*). **(C)** Reverse-transcription was carried out on RNA isolated from wildtype and CD63-transfected H69C cells. Using CD63-specific primers, PCR was performed on cDNA products using 1 µL, 2.5 µL, 5 µL and 7.5 µL (*lanes 1-4, 5-8, 9-12 and 13-16 respectively*) of RT template from untransfected (*lanes 1-4*), vector control pcDNA (*lanes 5-8*), wildtype CD63-transfected (*lanes 9-12*) and CD63phe-transfected (*lanes 13-16*) H69C cells to amplify CD63 as described in the Materials and Methods.